VALIDATED BIOANALYTICAL METHOD DEVELOPMENT FOR THE DETERMINATION OF ALOSETRON IN HUMAN K2EDTA PLASMA BY USING LC-MS/MS

DISSERTATION

Submitted to

THE TAMIL NADU DR.M.G.R.MEDICAL UNIVERSITY,

CHENNAI.

In partial fulfilment for the award of the degree of

MASTER OF PHARMACY

In

(Pharmaceutical Analysis)

By

261230006

Under the Guidance of

Mrs., VIJAYANAGARAJAN, M.Pharm., (Ph.D).,

Assistant Professor, Department of Pharmaceutical Analysis

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

C.L.BAID METHA COLLEGE OF PHARMACY

CHENNAI – 600 097

APRIL-2014

Phone : 24960151, 24960425
E-mail : principal@clbaidmethacollege.com
Website : www.clbaidmethacollege.org

C.L. Baid Metha College of Pharmacy

An ISO 9001 - 2000 certified institution

Jyothi Nagar, Old Mahabalipuram Road
Thorapakkam, Chennai - 600 097.

Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai.
Approved by Pharmacy Council of India, New Delhi, and
All India Council for Technical Education. New Delhi.

SRI. VINOD KHANNA
Chairman

SRI. L.UDAYMETHA
Secretary&Correspondent

Dr.SHANTHA ARCOT, **M.Sc**.**(Pharm)**,**Ph.D**., **Dr.GRACERATHNAM,MPharm**.,**Ph.D**.,
HOD, Department of Pharmaceutical Analysis, Principal

CERTIFICATE

This is to certify that the project entitled **"VALIDATED BIOANALYTICAL METHOD DEVELOPMENT FOR THE DETERMINATION OF ALOSETRON IN HUMAN K2EDTA PLASMA BY USING LC-MS/MS"** submitted **by 261230006** in partial fulfilment for the award of degree of **Master of Pharmacy.** It was carried out at Ethics Bio Lab Pvt Ltd and at C.L. Baid Metha College of Pharmacy, Chennai-96. under the supervision of **Mrs.Vijayanagarajan, M.Pharm., Ph.D.,Assistant professor,** Department of pharmaceutical analysis during the academic year **2013-2014.**

Place: Chennai- 97
Date:

Mrs.VijayanagarajanM.Pharm,(Ph.D),
Department of Pharmaceutical Analysis.
C.L.BaidMetha College of Pharmacy, Chennai-97

Phone : 24960151, 24960425
E-mail : principal@clbaidmethacollege.com
Website : www.clbaidmethacollege.org

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SRI. L. UDAY METHA
Secretary & Correspondent

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Place: Chennai- 97
Date:

Mrs.Dr.A.Shantha, B.Pharm, M.Sc.(Pharm).,Ph.D.,
HOD, Department of Pharmaceutical Analysis.
C.L.BaidMetha College of Pharmacy, Chennai-97

Phone : 24960151, 24960425
E-mail : principal@clbaidmethacollege.com
Website : www.clbaidmethacollege.org

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Chairman

SRI. L. UDAY METHA
Secretary & Correspondent

Dr.SHANTHA ARCOT, **M.Sc**.**(Pharm)**,**Ph.D**., **Dr.GRACERATHNAM,MPharm**.,**Ph.D**.,
HOD, Department of Pharmaceutical Analysis, Principal

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Place**:** Chennai -97
Date:

Mrs.Dr. GRACE RATHNAM, M.Pharm, Ph.D.,
Principal, Professor and HOD, Pharmaceutics,
C .L.BaidMetha College of Pharmacy, Chennai - 97.

DECLARATION

The thesis entitled **"VALIDATED BIOANALYTICAL METHOD DEVELOPMENT FOR THE DETERMINATION OF ALOSETRON IN HUMAN K2EDTA PLASMA BY USING LC-MS/MS"** was carried out by me in Department of Pharmaceutical Analysis, C.L. Baid Metha College of Pharmacy, Chennai – 97 during the academic year 2013-2014. The work embodied in this thesis is original, and is not submitted in part or full for any other degree of this or any other University.

Place: Chennai- 97

Date:

[Reg.No: 261230006]

DEPT OF PHARMACEUTICAL ANAYLSIS

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My humble thanks to the almighty, who gave me strength and confidence all along.

Place: Chennai -97 **Reg. No: 261230006**

Date: **Dept. of. PHARMACEUTICAL ANALYSIS**

Dedicated to

Parents,

Brothers and Friends

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LIST OF ABBREVIATIONS

%	:	Percentage
°C	:	Degree Celsius
µg	:	Micro gram
µL	:	Micro litre
µm	:	Micro meter
AQS.STD	:	Aqueous Standard
BA	:	Bioanalytical
BM	:	Bioanalytical Method
BTS	:	Bench Top Stability
CDS	:	Chromatographic data processing software
Conc.	:	Concentration
CS	:	Calibration Standards
CV	:	Coefficient of variation
DQC	:	Dilution Quality Control
EBL	:	Ethics Bio Lab Private Limited
ESI	:	Electro Spray Ionization
FT	:	Freeze-Thaw
GR	:	General Reagent
HQC	:	High Quality Control sample
ID	:	Identification
IMQC	:	Intermediate Quality control sample
ISTD	:	Internal Standard

K_2EDTA	:	Di Potassium Ethylene Diamine Tetra Acetate
LC/MS/MS	:	Liquid Chromatography/Mass Spectrometry/Mass Spectrometry
LLOQC	:	Lower Limit of Quality Control
LQC	:	Low Quality Control
m/z	:	Mass-to-charge ratio
mg	:	Milli gram
min	:	Minutes
mL	:	Milli litre
mm	:	Milli meter
mM	:	Milli molar
MQC	:	Medium Quality Control
NA	:	Not Applicable
ng	:	Nano gram
No.	:	Number
PA	:	Precision and Accuracy
QA	:	Quality Assurance
QC	:	Quality Control
r^2	:	Coefficient of determination
Rt	:	Retention Time
SOP	:	Standard Operating Procedure
SS	:	Spiking Solution
STD	:	Standard
ULOQ	:	Upper Limit of Quantification
UPLC	:	Ultra Performance Liquid Chromatography

V	:	Voltage
Vol.	:	Volume
Wt.	:	Weight

INTRODUCTION

INTRODUCTION

This thesis deals with the development and validation of Bioanalytical assay method used for the estimation of Alosetron in biological fluids. Before discussing the experimental results a brief introduction for method development, biopharmaceutical analysis and preliminary treatment of biological samples, extraction procedures for drugs and metabolites from biological samples and estimation of drugs in biological sample by LC-MS/MS for Alosetron.

Bio-availability and bio-equivalence studies require very precise and accurate assay methods that are well validated to quantify drugs in biological samples. The assay methods have to be sensitive enough to determine the biological sample concentration of the drug and/or its metabolite(s) for a period of about five elimination half-life after dosage of the drug. The assay methods also have to be very selective to ensure reliable data, free from interference of endogenous compounds and possible metabolites in the biological samples. In addition, methods have to be as robust and cost effective as possible, making of particular importance to bioequivalence studies. Above all, the assay methods must be able to withstand the scrutiny of national drug registration authorities who judge them on the basis of criteria established by international consensus.

Bioanalytical chemistry is the qualitative and quantitative analysis of drug substances in biological fluids (mainly plasma and urine) or tissue. It plays a significant role in the evaluation and interpretation of bioavailability, bioequivalence and pharmacokinetic data[1]. The main phases that comprise bioanalytical services are,

- Method development,
- Method validation,
- Sample analysis (method application).

Owing to increased interdependence among countries in recent times it has become necessary for results of many methods to be accepted internationally. Consequently, to assure common level of quality, the need for and use of validated methods has increased [2].

Whatever way the analysis is done it must be checked to see whether it does what it was intended to do; i.e. it must be validated. Each step in the method must be investigated to determine the extent to which environment, matrix, or procedural variables can affect the estimation of analyte in the matrix from the time of collection up to the time of analysis [3].

A full validation requires a high workload and should therefore only start when promising results are obtained from explorative validation performed during the method development phase. The process of validating a method cannot be separated from the actual development of method

conditions, because the developer will not know whether the method conditions are acceptable until validation studies are performed[2]. Method development clears the way for the further processes on the validation stage. It must be recognized that proper validation requires a lot of work. However, this effort is repaid by the time saved when running the method routinely during sample analysis.

1.0 BIOPHARMACEUTICAL ANALYSIS

1.1. NEED FOR BIOPHARMACEUTICAL ANALYSIS

Methods of measuring drugs in biological media are increasingly important related to following;

- Bioavailability and Bioequivalence Studies,
- New Drug Development,
- Clinical Pharmacokinetics,
- Research in Basic Biomedical and Pharmaceutical Sciences.

1.2. ASSAY OF DRUGS AND THEIR METABOLITES

A number of allusions have been made to methods that distinguish drugs from their metabolites. Drug metabolism reactions can be divided into phase I and phase II categories. Phase I typically involves oxidation, reduction, and hydrolysis reactions. In contrast, phase II transformations entail coupling or condensation of drugs.This involves glucoronidation, sulfation, aminoacid conjugation, acetylation, and methylation. Except for reduction processes, most phase I and phase II reactions yield metabolites that are more polar and hence more water soluble than the parent drug. Assays must distinguish between drug and its metabolites. If this fact is ignored, erroneous data may be generated.

1.3. ANALYSIS OF DRUGS IN VARIOUS BIOLOGICAL MEDIA

The most common samples obtained for biopharmaceutical analysis are blood, plasma and urine. Faeces are also utilized, especially if the drug or metabolite is poorly absorbed or extensively excreted in the bile. Other media that can be utilized includes saliva, and tissue.

The choice of sampling media is determined largely by the nature of the drug study. All most the drug levels in a clinical pharmacokinetic study demand the use of blood, urine, and possibly saliva.

A bioavailability study may require drug level data in blood and/or urine whereas a drug identification or drug abuse problem may be solved with any one type of biological sample.

Detection of a drug or its metabolite in biological media is usually complicated by the matrix. Because of this, various types of cleanup procedures involving techniques such as solvent extraction and chromatography are employed to effectively separate drug components from endogenous biologic material. The ultimate sensitivity and selectivity of the assay method may be limited by the efficiency of the cleanup methodology.

If the blood is allowed to clot and is then centrifuged, about 30 to 50% of the original volume is collected as serum (upper level). Thus, plasma generally is preferred because of its greater yield from blood. Blood, serum or plasma samples can be utilized for drug studies and may require protein denaturation steps before further manipulation.

If plasma or serum is used for the procedure, the fresh whole blood should be centrifuged immediately at 4000rpm for approximately 5 to 10 min, and the supernatant should be transferred by means of a suitable device, such as a Pasteur pipette, to a clean container of appropriate size for storage.

Urine is easiest to obtain from the patient and also permits collection of large and frequently more concentrated samples. The lack of protein in a healthy individual's urine obviates the need for denaturation steps. Because urine samples are readily obtained and often provide the greatest source of metabolites, they are frequently analyzed in drug metabolism studies.

With humans, faeces are collected in an aluminium foil pan placed under a toilet seat. Once collected, the foil is folded around the material and the sample lyophilized. Faecalspecimens contain high protein content, and difficulties arise in their handling and analysis (even after Lyophilization) because of the large ratio of solid mass to drug. Denaturation of protein is usually required before further manipulations are begun.

Saliva and biological media obtained from humans when constant ratio between plasma and salivary levels of certain drugs exists via non invasive sampling techniques. Saliva is advantageous in drug studies done with children. Although the concentrations of drugs in saliva are rarely equal to those in plasma, a constant ratio (over an effective therapeutic range) permits calculation of plasma levels based on salivary analysis.

Separation or isolation of drugs and metabolites from biologic samples is performed in order to partially purify a sample. In this manner, an analyst can obtain the selectivity and sensitivity needed to detect a particular compound and can do so with minimum interference from components

of the more complex biological matrix. The number of steps in a separation procedure should be kept to a minimum to prevent loss of drug or metabolite. Sometimes, the separation steps are preceded by a sample pretreatment.

1.4. STORAGE REQUIREMENTS FOR BIOLOGICAL SAMPLES

In order to avoid decomposition or other potential chemical changes in the drugs to be analyzed, biological samples should be frozen immediately upon collection and thawed before analysis. When drugs are susceptible to plasma esterases, the addition of esterase inhibitors, such as sodium fluoride, to blood samples immediately after collection helps to prevent drug decomposition .

When collecting and storing biological samples, the analyst should be wary of artifacts from tubing or storage vessels that can contaminate the sample. For example, plastic-ware frequently contains the high boiling liquid bis (2-ethylhexyl) phthalate; similarly, the plunger-plugs of vacutainers are known to contain tri-butoxyethylphosphate, which can interfere in certain drug analysis.

1.5. PRELIMINARY TREATMENT OF BIOLOGICAL SAMPLES

In most cases, preliminary treatment of a sample is needed before the analyst can proceed to the measurement step. Analysis is required for drug in samples as diverse as plasma, urine, faeces, saliva, bile, sweat, and seminal fluid. Each of these samples has its own set of factors that must be considered before an appropriate pretreatment method can be selected. Such factors as texture and chemical composition of the sample, degree of drug-protein binding, chemical stability of the drug, and types of interferences can affect the final measurement step.

1.5.1. PROTEIN PRECIPITATION OR DENATURATION

Biological materials such as plasma, faeces, and saliva contain significant quantities of protein, which can bind a drug. The drug may have to be freed from protein before further manipulation. Protein denaturation is important, because the presence of proteins, lipids, salts, and other endogenous materials in the sample can cause rapid deterioration of HPLC columns and also interfere the assay.

Protein denaturation procedures include the use of tungstic acid, ammonium sulfate, heat, alcohol, trichloroacetic acid, and perchloric acid.

Methanol and acetonitrile frequently have been used as protein denaturants of biological samples. Methanol sometimes is preferred because it produces a flocculent precipitate and not the

gummy mass obtained with acetonitrile. Methanol also gives a clearer supernatant and may prevent the drug entrapment that can be observed after acetonitrile precipitation.

Ultrafiltration and dialysis procedures also have been used to remove proteins from biological fluids. These procedures are not widely used because they are slow.

1.5.2. HYDROLYSIS OR CONJUGATES

The presence-of drug metabolites as conjugates, such as glucuronides and sulfates, in biological samples cannot be ignored. The effect of a drug depends to a considerable extent on the biotransformation that occurs in the human body. Therefore, it may be important to isolate the actual conjugates. Samples containing either glucuronideacetals or sulfate esters are usually pretreated using enzymatic or acid hydrolysis. The unconjugated metabolites that result from the hydrolysis procedure are less hydrophilic than their conjugates and usually can be extracted from the biological matrix.

A nonspecific acid hydrolysis can be accomplished by heating a biological sample for 30 min at 90 to 100°C in 2 to 5N hydrochloric acid. Upon cooling, the pH of the sample can be adjusted to the desired level and the metabolite removed by solvent extraction. Particularly stable conjugates sometimes require hydrolysis in an autoclave.

1.5.3. HOMOGENIZATION

For samples containing insoluble protein, such as muscle or other related tissues, a homogenization or solubilizing step using 1N hydrochloric acid may be required before treating the sample further. For gelatinous samples such as seminal fluid or sputum, liquefaction is achieved via sonication. A solid sample such as faeces can be homogenized with a minimum amount of methanol. Homogenization is usually performed with a blade homogenizer (e.g., Warring Blender).

1.6 EXTRACTION PROCEDURES FOR DRUGS AND METABOLITES FROM BIOLOGICAL SAMPLES

After pre treatment of biological material, the next step is usually the extraction of the drugs from the biological matrix. All separation procedures use one or more treatments of matrix-containing solute with some fluid. As extracting solvents are liquid and the biological sample solid (e.g., lyophilized faeces), it is an example of liquid-solid extraction. If the extraction involves two liquid phases, it is an example of liquid-liquid extraction.

1.6.1 LIQUID-SOLID EXTRACTION

Liquid - solid extractions occur between a solid phase and a liquid phase, either phase may initially contain the drug substance. Among the solids that have been used successfully in the extraction (usually via adsorption) of drugs from liquid samples are XAD-2 resin, charcoal, alumina, silica gel, and aluminum silicate. Sometimes the drugs are contained in a solid phase, such as in lyophilized specimens. Liquid-solid extraction is often particularly suitable for polar compounds that would otherwise tend to remain in the aqueous phase. The method could also be useful for amphoteric compounds that cannot be extracted easily from water.

Factors governing the adsorption and elution of drugs from the resin column include solvent polarity; flow rate of the solvent through the column, and the degree of contact between the solvent and with the resin beds.

In the adsorption process, the hydrophobic portion of the solute that has little affinity for the water phase is preferentially adsorbed on the resin surface while the hydrophilic portion of the solute remains in the aqueous phase. Alteration in the lipophilic / hydrophilic balance within the solute or solvent mix, and not within the resin, affects adsorption of the solute.

Biological samples can be prepared for cleanup by passing the sample through the resin bed where drug (metabolite) components are adsorbed and finally eluted with an appropriate solvent. The liquid-solid extraction method provides a convenient isolation procedure for blood samples, thus avoiding solvent extraction, protein precipitation, drug losses, and emulsion formulation. It is possible; however, that strong drug-protein binding could prevent sufficient adsorption of the drug to resin.

DEHYDRATION METHODS

An aqueous biological sample is treated with a sufficient quantity of anhydrous salt (sodium or magnesium sulfate) to create a "dried" mix. This mix is then extracted with a suitable organic solvent to remove the desired drug or metabolite.

1.6.2. LIQUID-LIQUID EXTRACTION

Liquid-liquid extraction is probably the most widely used technique because the analyst can remove a drug or metabolite from larger concentrations of endogenous materials that might interfere with the final analytical determination.

The technique is simple, rapid, and has a relatively small cost factor per sample.

The extract containing the drug can be evaporated to dryness, and the residue can be reconstituted in a smaller volume of a more appropriate solvent. In this manner, the sample

becomes more compatible with a particular analytical methodology in the measurement step, such as a mobile phase in LCMS/MS determinations.

The extracted material can be reconstituted in small volumes (e.g., 100 to 500 µl of solvent), thereby extending the sensitivity limits of an assay. It is possible to extract more than one sample concurrently. Quantitative recoveries (90% or better) of most drugs can be obtained through multiple or continuous extractions.

Partitioning or distribution of a drug between two possible liquid phases can be expressed in terms of a partition or distribution coefficient, usually called partition coefficient is constant only for a particular solute, temperature, and pair of solvents used. By knowing the P value for the extracted drug and the absolute volumes of the two phases to be utilized, the quantity of drug extracted after a single extraction can be obtained. In multiple extractions methodology, the original biological sample is extracted several times with fresh volumes of organic solvent until as much drug as possible is obtained. Because the combined extracts now contain the total extracted drug, it is desirable to calculate the number of extractions necessary to achieve maximum extraction.

FACTORS AFFECTING THE PARTITION COEFFICIENT

Factors that influence partition coefficient and hence recovery of drugs in liquid-liquid extraction are choice of solvent, pH, and ionic strength of the aqueous phase. In almost all cases, one of the liquid phases is aqueous because of the nature of a biological sample. The second liquid is selected by the analyst. It is highly desirable to select an organic solvent that shows greater affinity for the drug analyzed, yet leaves contaminants or impurities in the aqueous or biological phase. The solvent should be immiscible with an aqueous phase, should have less polarity than water, and should solubilize the desired extractable compound to a large extent. It should also have a relatively low boiling point so that it can be easily evaporated if necessary. Other considerations are cost, toxicity, flammability, and the nature of the solvent. If larger numbers of samples are to be extracted, the volume of solvent needed per sample can affect the overall cost of the assay procedure.

It is generally accepted that diethyl ether and chloroform are the solvents of choice for acidic and basic drugs, respectively, especially when the identity of the drugs in the samples are unknown. In these cases, any chemically neutral drugs are extracted into either solvent depending on their relative partition tendencies.

Proper pH adjustment of a biological sample permits quantitative conversion of an ionized drug to an un-ionized species, which is more soluble in a nonpolar solvent and therefore, extractable from an aqueous environment. In analysis, do determine a known drug or metabolite,

the proper pH for extraction can be calculated from the Henderson-Hassel Balch equation using the pKa of the compound. If the species to be analyzed is unknown, the pH must be approximated based on the chemical nature of the suspected agent.

Third Factor influencing extractability of drugs from biological samples is ionic strength. Addition of highly water-soluble ionized salts, such as sodium chloride, to an aqueous phase creates a high degree of interaction between the water molecules and the inorganic ions in solution. Fewer water molecules are free to interact with an unionized drug. Therefore, the solubility of the drug in the aqueous phase decreases, thereby increasing the partitioning or distributing in favor of the non-polar or organic phase. The technique is commonly called "salting out."

Either mechanical or manual tumbling, rocking, or vigorous shaking of the samples can accomplish mixing of the aqueous organic phases . The percent recovery of a drug vs. time and/or type of mixing should be investigated for each biological sample. In many cases, vigorous shaking of a sample should be avoided because it leads to emulsification, which can be intractable for centrifugation. Emulsification is often observed when organic solvents are used at basic pH whereas certain organic solvents such as n-hexane and diethyl ether are less emulsion-prone.

Certain types of amphoteric drugs or drugs that possess extreme water solubility are not amenable to classic solvent extraction. In these cases, other types of analytical methodology such as ion-pairing must be adopted.

The technique of back-extraction can be applied with success to the analysis of drugs in biological samples. The purpose of the methodology is to further purify an extract by removing either drug or impurities by additional extractions.

1.7. CHROMATOGRAPHIC METHODS

The presence of metabolites or more than one drug in a biological sample usually demands a more sophisticated separation for their measurement especially, when two or more drugs are of similar physical and chemical nature. Chromatography is a separation technique that is based on differing affinities of a mixture of solutes between at least two phases. The result is a physical separation of the mixture into its various components. The affinities or interactions can be classified in terms of a solute adhering to the surface of a polar solid (adsorption), a solute dissolving in a liquid (partition), and a solute passing through or impeded by a porous substance based on its molecular size (exclusion).

1.7.1. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

HPLC is directly derived from classic column chromatography in that a liquid mobile phase is pumped under pressure rather than by gravity flow through a column filled with a stationary phase. This has resulted in a sharp reduction in separation time, narrower peak zones, and improved resolution. The mobile phase is placed in a solvent reservoir for pumping into the system. In the case of liquid-solid HPLC, solvents are chosen from the elutropic series. A solvent system is usually degassed by vacuum treatment or sonication before use.

1.7.2. LIQUID CHROMATOGRAPHY MASS SPECTROMETRY

Liquid chromatography is a fundamental separation technique in the life sciences and related fields of chemistry. Unlike gas chromatography, which is unsuitable for nonvolatile and thermally fragile molecules, liquid chromatography can safely separate a very wide range of organic compounds, from small-molecule drug metabolites to peptides and proteins.

Traditional detectors for liquid chromatography include refractive index, electrochemical, fluorescence, and ultraviolet-visible (UV-Vis) detectors. Some of these generate two dimensional data; that is, data representing signal strength as a function of time. Others, including fluorescence and diode array UV-Vis detectors, generate three-dimensional data. Three-dimensional data include not only signal strength but spectral data for each point in time.

Mass spectrometers also generate three dimensional data. In addition to signal strength, they generate mass spectral data that can provide valuable information about the molecular weight, structure, identity, quantity, and purity of a sample.

Mass spectral data add specificity that increases confidence in the results of both qualitative and quantitative analysis.

For most compounds, a mass spectrometer is more sensitive and far more specific than all other LC detectors. It can analyze compounds that lack a suitable chromophore. It can also identify components in unresolved chromatographic peaks, reducing the need for perfect chromatography.

Some mass spectrometers have the ability to perform multiple steps of mass spectrometry on a single sample. They can generate a mass spectrum, select a specific ion from that spectrum, fragment the ion, and generate another mass spectrum; repeating the entire cycle many times. Such mass spectrometers can literally deconstruct a complex molecule piece by piece until its structure is determined.

Mass spectral data complements data from other LC detectors. While two compounds may have similar UV spectra or similar mass spectra, it is uncommon for them to have both.

1.8. ESTIMATION OF DRUGS IN BIOLOGICAL SAMPLES BY LC-MS/MS

MS has emerged as an ideal technique for the identification of such structurally diverse metabolites. When coupled with online HPLC the technique is extremely robust, rapid, sensitive, and easily automated. Not surprisingly, LC/MS/MS have become the methods of choice for pharmacokinetic studies, yielding concentration versus time data for drug compounds from in vivo samples such as plasma.

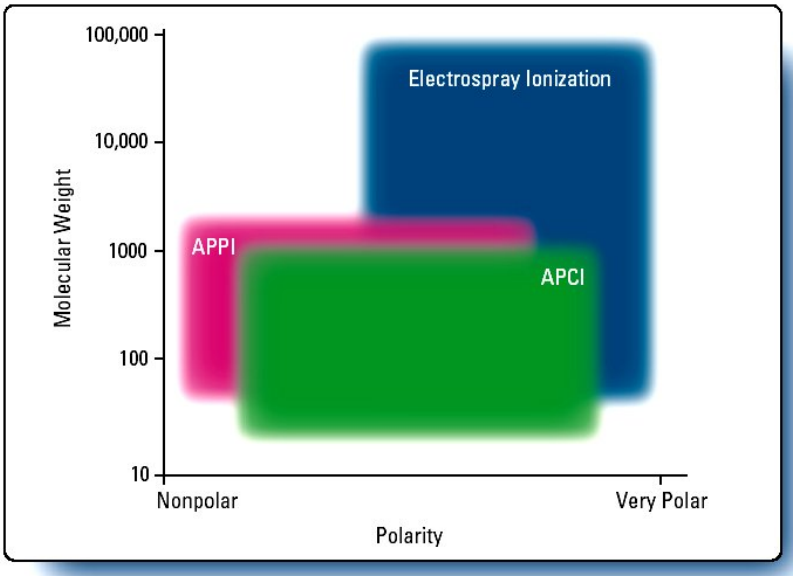
LC-MS instrument consist of three major components

- LC (to resolve a complex mixture of components)
- An interface (to transport the analyte in to the ion source) of a mass spectrometer
- Mass spectrometer (to ionize and mass analyze the individually resolved components)

Reverse phase (RP) HPLC is a widely pretended mode of chromatography and is a major contributing factor to advances made in several areas of pharmaceutical science. Mobile phase composition is a very critical in achieving selectivity in RP-HPLC separation. Although a large number of buffer system have been used in conventional RP-HPLC, only the volatile ion paring reagent can be used in LC-MS analysis.

1.8.1. IONIZATION TECHNIQUES

Interface is used for transporting the analyte into the ion source of a mass spectrometry. The different types of ionization techniques are ESI, APCI, APPI most commonly used ionization techniques.

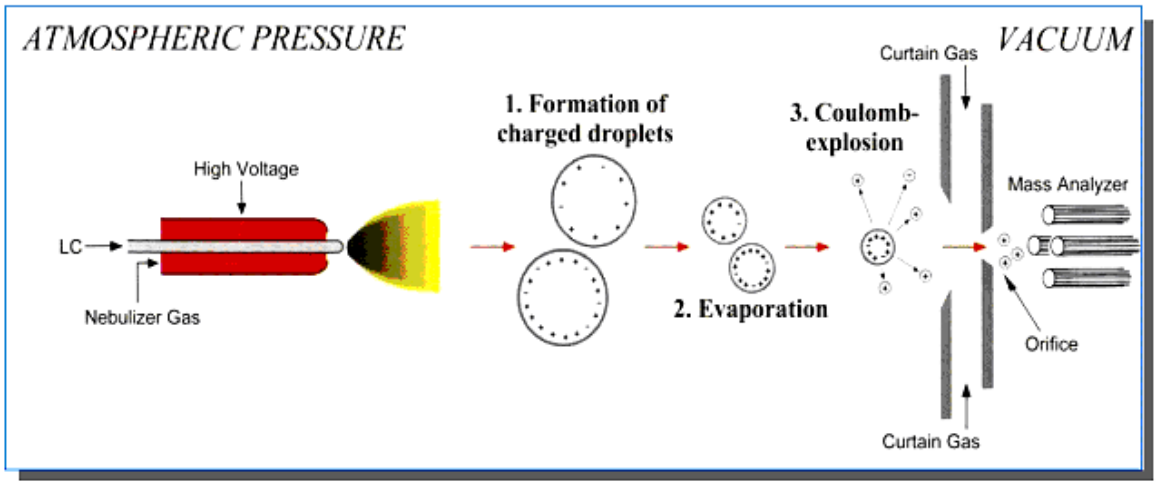


1.8.1.1. ELECTROSPRAY IONIZATION (Turbo spray)

Electrospray relies in part on chemistry to generate analyte ions in solution before the analyte reaches the mass spectrometer. The LC eluent is sprayed (nebulized) into a chamber at atmospheric pressure in the presence of a strong electrostatic field and heated drying gas. The electrostatic field causes further dissociation of the analyte molecules.

The heated drying gas causes the solvent in the droplets to evaporate. As the droplets shrink, the charge concentration in the droplets increases. Eventually, the repulsive force between ions with like charges exceeds the cohesive forces and ions are ejected (desorbed) into the gas phase. These ions are attracted to and pass through a capillary sampling orifice into the mass analyzer.

Some gas-phase reactions, mostly proton transfer and charge exchange, can also occur. Between the times, ions are ejected from the droplets and they reach the mass analyzer.

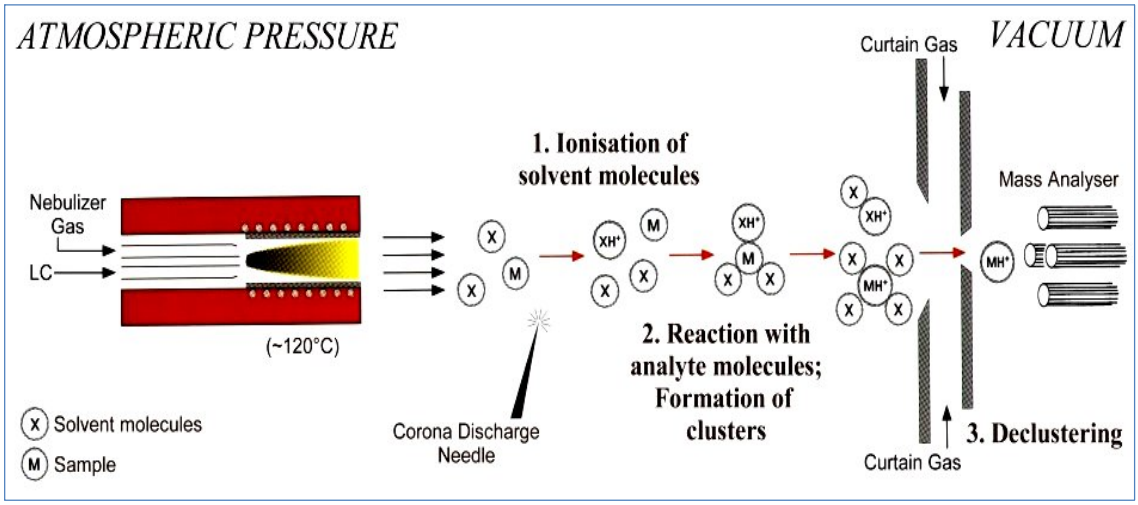


1.8.1.2. ATMOSPHERIC PRESSURE CHEMICAL IONIZATION

In APCI, the LC eluent is sprayed through a heated (typically 250°C – 400°C) vaporizer at atmospheric pressure. The heat vaporizes the liquid. The resulting gas-phase solvent molecules are ionized by electrons discharged from a corona needle. The solvent ions then transfer charge to the analyte molecules through chemical reactions (chemical ionization).

The analyte ions pass through a capillary sampling orifice into the mass analyzer. APCI is applicable to a wide range of polar and nonpolar molecules. It rarely results in multiple charging so it is typically used for molecules less than 1,500μ. Due to this, and because it involves high temperatures, APCI is less well-suited than electrospray for analysis of large biomolecules that may

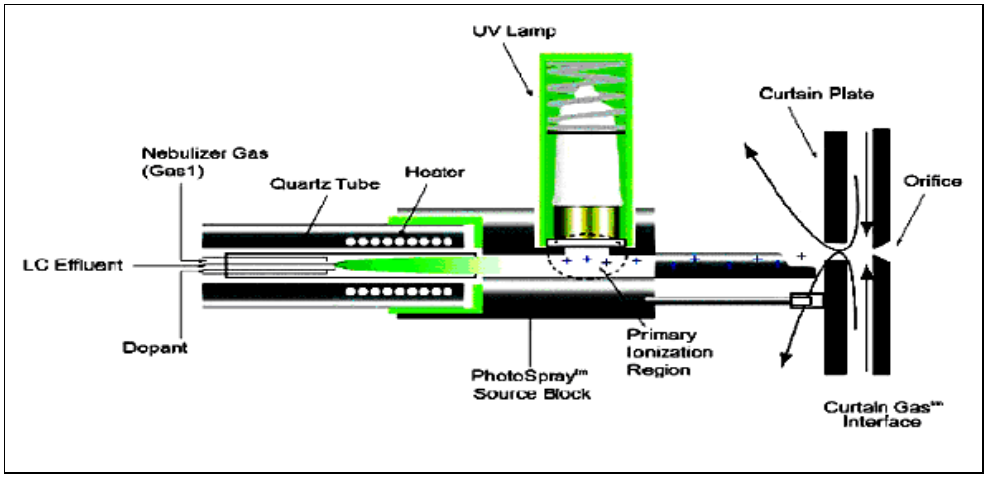
be thermally unstable. APCI is used with normal-phase chromatography more often than electrospray is because the analytes are usually nonpolar.



Atmospheric pressure photo ionization (APPI) for LC-MS/MS is a relatively new technique. As in APCI, a vaporizer converts the LC eluent to the gas phase. A discharge lamp generates photons in a narrow range of ionization energies. The range of energies is carefully chosen to ionize as many analyte molecules as possible while minimizing the ionization of solvent molecules. The resulting ions pass through a capillary sampling orifice into the mass analyzer.

APPI is applicable to many of the same compounds that are typically analyzed by APCI. It shows particular promise in two applications, highly nonpolar compounds and low flow rates (<100 µl/min), where APCI sensitivity is sometimes reduced.

In all cases, the nature of the analyte(s) and the separation conditions has a strong influence on which ionization technique: electrospray, APCI, or APPI will generate the best results. The most effective technique is not always easy to predict.



1.8.2. MASS ANALYZER (Quadrupole)

A quadrupole mass analyzer consists of four parallel rods arranged in a square. The analyte ions are directed down the center of the square. Voltages applied to the rods generate electromagnetic fields. These fields determine which mass-to-charge ratio of ions can pass through the filter at a given time. Quadrupoles tend to be the simplest and least expensive mass analyzers.

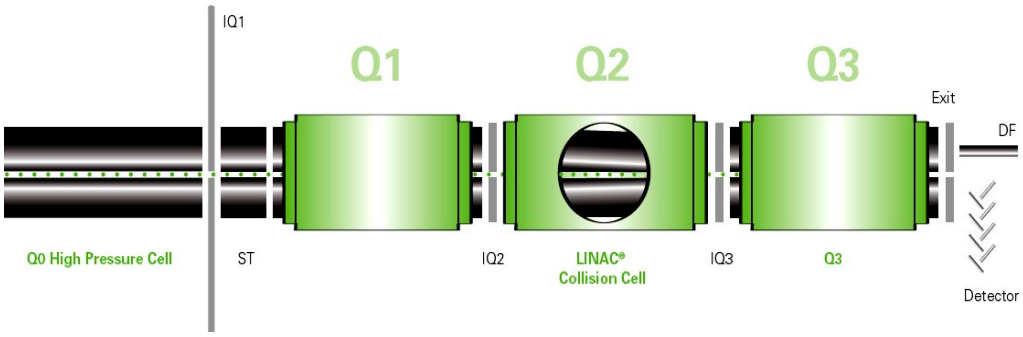
Quadrupole mass analyzers can operate in two modes:

- MRM Mode
- Scanning (scan) mode
- Selected ion monitoring (SIM) mode

In scan mode, the mass analyzer monitors a range of mass-to-charge ratios. In SIM mode, the mass analyzer monitors only a few mass to- charge ratios.

SIM mode is significantly more sensitive than scan mode but provides information about fewer ions. Scan mode is typically used for qualitative analyses or for quantitation when all analyte masses are not known in advance.

SIM mode is used for quantitation and monitoring of target compounds.



1.9. APPLICATIONS OF LC-MS/MS

- Peptide mapping
- Selective detection of compounds in a complex mixture
- Efficient analysis of biological samples
- To identify degradation products in stability studies
- Identification of metabolites
- Quantification of compounds in biological matrix.

1.10. QUANTITATIVE ANALYSIS

Three methods are generally used for quantitative analysis. They are the external standard method, the internal standard method and the standard addition method.

1.10.1 EXTERNAL STANDARD METHOD

The external standard method involves the use of a single standard or up to three standard solutions. The peak area or the height of the sample and the standard used are compared directly or the slope of the calibration curve based on standards that contain known concentrations of the compounds of interest.

1.10.2. INTERNAL STANDARD METHOD

A widely used technique of quantitation involves the addition of an internal standard to compensate for various errors. In this approach, a known compound of a fixed concentration is added to the known amount of samples to give separate peaks in the chromatograms, to compensate for the losses of the compounds of interest during sample pretreatment steps. Any loss of the component of interest will be accompanied by the loss of an equivalent fraction of internal standard. The accuracy of this approach obviously dependents on the structural equivalence of the compounds of interest and the internal standard.

The requirements for an internal standard must

- Give a completely resolved peak with no interferences,
- Elute close to the compound of interest,
- Behave equivalent to the compounds of interest for analysis like pretreatments, derivative formations, etc.,
- Be added at a concentration that will produce a peak area or peak height ratio of about unity with the compounds of interest,
- Not be present in the original sample,
- Be stable, unreactive with sample components, column packing and the mobile phase and
- Be commercially available in high purity.
- Free from Drug-drug intraction

The internal standard should be added to the sample prior to sample preparation procedure and homogenized with it. Response factor is used to determine the concentration of a sample

component in the original sample. The response factor (RF) is the ratio of peak areas of sample component (Ax) and the internal standard (ISTD) obtained by injecting the same quantity.

1.11. METHOD DEVELOPMENT

The method development and establishment phase defines the chemical assay.

A bioanalytical method is a set of all procedures involved in the collection, processing, storing, and analysis of a biological matrix for an analyte methods employed for quantitative determination of drugs and their metabolites in biological fluids are the key determinants in generating reproducible and reliable data that in turn are used in the evaluation and interpretation of bioavailability, bioequivalency and pharmacokinetics.

Method development involves evaluation and optimization of the various stages of sample preparation, chromatographic separation, detection and quantification. To start these works an extensive literature survey, reading work done on the same or similar analyte and summarizing main starting points for future work is of primary importance. Based on the information from the survey, the following can be done.

- Choice of instrument that is suitable for the analysis of analyte of interest.
- Choice of the column associated with instrument of choice, the detector and the mobile phase.
- Choice of internal standard, (It must have similar chromatographic properties of analyte.)
- Choice of extraction procedure, (which is time economical, gives the highest possible recovery without interference and has acceptable accuracy and precision.)

Another important issue in method development stage is the choice of internal versus external standardization. Internal standardization is common in bioanalytical methods especially with chromatographic procedures. For internal standardization, a structural or isotopic analogue of the analyte is added to the sample prior to sample pre-treatment and the ratio of the response of the analyte to that of the internal standard is plotted against the concentration. Another important point is that the tests performed at the stage of method development should be done with the same equipment that will actually be used for subsequent routine analysis. The differences found between individual instruments representing similar models from the same manufacturer is not surprising and should be accounted.

1.11.1. Optimization of chromatographic conditions

Optimization of liquid chromatography parameters in bio analytical method is mainly objected towards improvement of resolution, peak shape and removal of interference at analytes Rt.

In case of LC-MS/MS method because of highly specific and selective detection of analytes because of MRM detection mode, less effort are needed to for improvement of resolution.

Generally mobile phase optimization is always preferred prior to stationary phase optimization.

1.11.2. Selection of mobile phase

There are many more restrictions regarding the selection of eluents and eluent components for use with HPLC-MS methods compared with HPLC- UV methods. Essentially the buffer constituents have to be volatile so the usual standby, phosphate, is unacceptable, as are the other common eluent constituents such as ion- pair reagents, organic amines, etc. Whilst the use of these eluent components may give acceptable results (at least in the short term), they would rapidly result in fouling of the source of common atmospheric ionization interfaces. This would lead to loss in sensitivity and significant down-time as the source was repeatedly cleaned. The use of MS detection therefore necessitates a new way of thinking about HPLC eluent.

1.11.3. Organic Modifiers

Firstly a decision must be made regarding the use of methanol or acetonitrile. Methanol is slightly superior to acetonitrile with ESI since it gives marginally greater response. Methanol is also preferred since it is reported to give slightly better peak shape for basic compounds in RP- HPLC than acetonitrile and it is a better solvent for buffer salts.

1.11.4. Mobile Phase Buffering

Wherever acidic or basic samples are separated it is strongly advisable to control mobile phase pH by adding a buffer. The measurement of pH for a mobile that contains organic solvent is imprecise, because electrode response tends to drift. Consequently if a pH meter is to be used, it is strongly recommended that the pH of the buffer should be adjusted before adding organic. In selecting a particular buffer, several considerations should be kept in mind.

Buffer Capacity - Buffer capacity is determined by pH, buffer pKa and buffer concentration. As for the case of a sample compound buffer ionization occurs over a range in pH given by pKa ± 2. Only in this pH range can the buffer be effective in controlling pH. Therefore, to be on the safe side, the buffer selected for a particular separation should be used to control pH over a range ≈ pKa ± 1.0. For RPC separations, a buffer concentration of 10 to 50 mM is usually adequate. Higher buffer concentrations also may adversely affect the operation of HPLC systems constructed of stainless steel. A mobile phase with marginal buffer capacity will give less reproducible separations for compounds that are practically ionized at the pH of

the mobile phase. In this case, retention may change from run to run, and distorted peaks may result. Buffer solubility and stability, possible interaction with the equipment, sample, and/or column, and the volatility are also of interest for some applications.

1.11.5. Selection of column

When attempting a separation, a reverse-phase, bonded-hydrocarbon column should be selected first, because such columns have the widest applicability. Fully reacted monomeric bonded C8 packing represent a good compromise for reverse- phase separations, because these materials have moderate retention, good efficiency and stability, and a useful k' range for a wide variety of samples. C18 can be used for applications in which maximum retention and sample size is required. C18 packing sometimes also exhibit superior characteristics for compounds that have higher water solubility. Shorter-chain bonded hydrocarbon phases are useful in applications involving very strongly retained solutes, or to improve selectivity by the use of the higher concentrations of water required in the mobile phase for these packing.

As in LLC with mechanically held stationary liquids, retention in normal phase BPC increases with the polarity of the bonded-stationary phase. Depending on the organic functionality, polar BPC packings show significant selectivity differences when compared to bare silica packings and to each other and some polar bonded-phase packings are actually more retentive than bare silica.

Normal-phase BPC columns can be used as an alternative to adsorption chromatography and one of the most versatile of these materials is the nitrile (CN or cyano-) bonded-phase materials. Separation of very polar (including water-soluble) samples may require packings such as diol- or amino-BPC packings. However, if the sample itself is aqueous, a reverse- phase system should be attempted first, because this approach offers greater sampling convenience.

1.11.6. Selection of internal standard:

An internal standard at a known concentration is normally added to plasma samples and is utilized to diagnose several potential variations that can occur during sample preparation and ongoing analysis.

The structure of the internal standard should be similar to the drug of interest. If this is not possible other standards can be used.

Requirements for a proper internal standard:

- Well resolved from the compound of interest and other peaks.

- Similar retention to the Analyte.
- Should not be in the original sample.
- Should mimic the Analyte in any sample preparation steps.
- Does not have to be chemically similar to Analyte.
- Commercially available in high purity.
- Stable and uncreative with sample or mobile phase.
- Should have a similar detector response to the Analyte for the concentration used.

1.12. METHOD VALIDATION

The search for the reliable range of a method and continuous application of this knowledge is called validation. It can also be defined as the process of documenting that the method under consideration is suitable for its intended purpose.

Method validation involves all the procedures required to demonstrate that a particular method for quantitative determination of the concentration of an analyte (or a series of analytes) in a particular biological matrix is reliable for the intended application. Validation is also a proof of the repeatability, specificity and suitability of the method.

Bioanalytical methods must be validated if the results are used to support the registration of a new drug or a new formulation of an existing one. Validation is required to demonstrate the performance of the method and reliability of results. If a bioanalytical method is claimed to be for quantitative biomedical application, then it is important to ensure that a minimum package of validation experiments has been conducted and yields satisfactory results.

The guideline for industry by FDA states that the fundamental parameters of validation parameters for a bioanalytical method validation are accuracy, precision, selectivity, sensitivity, reproducibility and stability. Typical method development and establishment for bioanalytical method includes determination of (1) selectivity, (2) accuracy, (3) precision, (4) recovery, (5) calibration curve, and (6) stability.

For a bioanalytical method to be considered valid, specific acceptance criteria should be set in advance and achieved for accuracy and precision for the validation of the QC samples.

Validations are subdivided into the following three categories:

1.12.1. FULL VALIDATION

This is the validation performed when developing and implementing a bioanalytical method for the first time. Full validation should be performed to support pharmacokinetic, bioavailability, and bioequivalence and drug interaction studies in a new drug application (NDA).

1.12.2. PARTIAL VALIDATION

Partial validations are performed when modifications of already validated bioanalytical methods are made. Partial validation can range from as little as one intra-assay and precision determination to a nearly full validation. Some of the typical bioanalytical method changes that fall into this category include bioanalytical method transfer between laboratories or analyst, change in methodology, change of matrix within species, change of species within matrix. The decision of which parameters to be revalidated depend on the logical consideration of the specific validation parameters likely to be affected by the change made to the bioanalytical method.

1.12.3. CROSS VALIDATION

Cross validation is a comparison of validation parameters when two or more bioanalytical methods are used to generate data within the same study or across different studies. An example of cross validation would be a situation when the original validated bioanalytical method serves as the reference and the revised bioanalytical method is the comparator.

1.12.4. VALIDATION PARAMETERS

The following experimental design is drawn in order to prove the test method is capable to yield consistent, reliable and reproducible results within the pre-determined acceptance limits.

Acceptance criteria for the validation parameters are specified in individual experimental design.

Observations and results were recorded in individual method validation data sheets.

The following parameters have been validated.

1. carryover test
2. Selectivity
3. sensitivity
4. matrix effect
5. Linearity
6. Precision and Accuracy

7. Recovery

8. Dilution integrity

9. Ruggedness

10. Stabilities

    10.1 Room temperature stability

    10.2. Refrigerator stock solution stability

    10.3 Bench top stability

    10.4 Auto sampler stability

    10.5 Long term stability

    10.6 Freeze thaw stability

    10.7 Wet Extract Stability

11. Re-injection stability

12. Concomitant Drug Effect

Acceptance Criteria:

- Precision: The precision calculated for Low and High QC concentrations should be within 15% and 20% for the LLOQ QC Concentrations.
- Accuracy: The accuracy calculated for Low and High QC concentrations should be within ± 15 % and ± 20 % for the LLOQ QC Concentration of the nominal value.
- At least 67% of the QC samples should be within 15% of their respective nominal values except at LLOQ QC where it should be within 20% of the nominal values. 33% of the QC samples (not all replicates at the same concentration) can be out side the above acceptance limits. At least 50% of QC samples at each concentration level should be within ±15% of their respective nominal value except at LLOQ QC where it must be within ± 20 % of the nominal value.

1.13. SAFETY PRECAUTIONS

Always wear protective clothing, particularly disposable gloves and masks during handling of drug samples. Gloves should be removed in such a way that the skin does not come into contact with external surface of the glove.

If biological matrix is spilled, it should be cleaned up immediately with 4% hypochlorite solution. Hands should be washed with soap and water before leaving the laboratory or whenever contaminated. Broken glassware should not be handled directly by hand. Pick up by mechanical means such as brush, dustpan, tongs or forceps.

1.14. DATA PROCESSING

The chromatograms were acquired using the computer based Analyst 1.4.2 software. The data was processed by peak area ratio method using same software. The concentration of the unknown was calculated from the following equation using regression analysis of spiked calibration of standard with the reciprocal of the square of the drug to internal standard concentration ratio as a weighting factor [1/(concentration ratio) ^{2}].

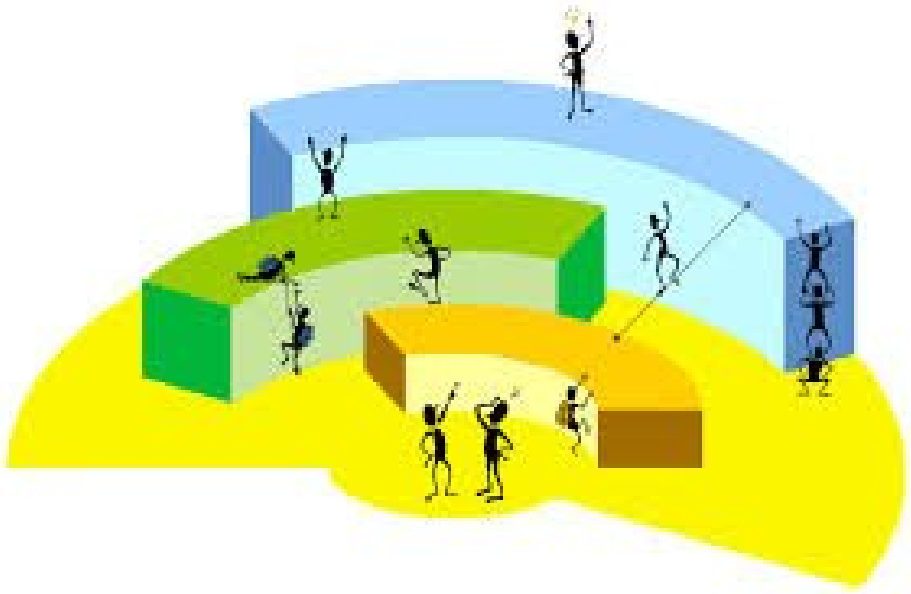
$$y = mx + c$$

Where, y = peak area ratio of Naproxen to internal standard

m = slope of the calibration curve

x = concentration ratio of Naproxen to internal standard

c = y-axis intercept of the calibration curve.



AIM AND OBJECTIVE

AIM AND OBJECTIVE

AIM:

The aim of this study is to develop and validate a specific bioanalytical method by LC-MS/MS for the estimation of Alosetron in K2EDTA human plasma using Alosetron D_3 as internal standard

OBJECTIVE:

Methods of measuring drugs in biological media are increasingly important due to problems related to bioavailability and bioequivalence, new drug development, drug abuse, clinical pharmacokinetics, and drug research are highly dependent on accurately measured drugs in biological samples.

For the estimation of the drugs present in the biological fluid, LCMS/MS method is consider to be more suitable since this is a powerful and rugged method. It is also extremely specific, linear, precise, accurate, sensitive and rapid.

Currently there is a need in the pharmaceutical environment to develop Bio-analytical methods for the determination of Alosetron in human plasma. The developed method could then be applied to clinical trials to obtain accurate pharmacokinetic parameters in human plasma.

Already HPLC-UV, LC-MS / MS, and GC-MS methods have been reported for Alosetron in various Biological media. Some of these methods use complicated extraction instruments, long and tedious extraction procedures, and large amounts of solvents or biological fluids for extraction while other methods have a long turnaround time during analysis

The main objective of this work is to develop rapid, selective and sensitive HPLC-UV and LC-MS / MS methods that have short and simple extraction procedures, consume small amounts of solvent and biological fluid for extraction and a short turn-around time.

1. Litrature survey

a) Alosetron – category, molecular formula, molecular weight, chemistry, Physiochemical properties, pharamacological & pharmaceutical properties.

b) Search for analytical methods if any (HPLC, LCMS/MS)

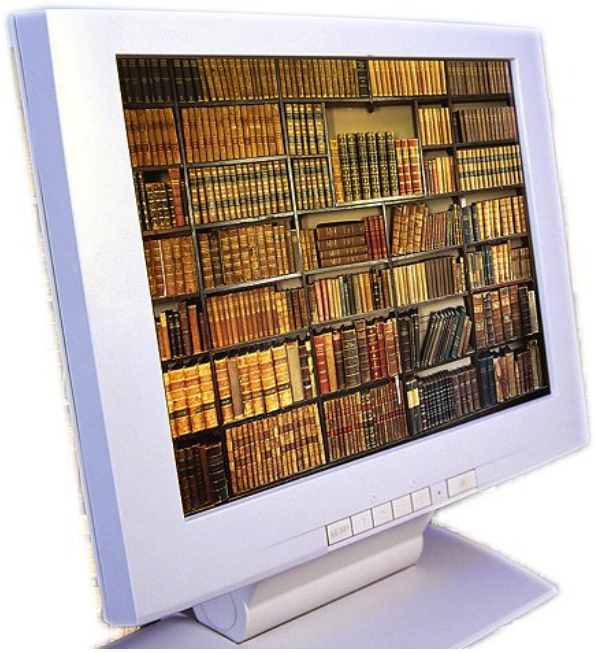
2. Method Development

- Stock solution preparation and tunning of analyte.,
- Optimization of chromatographic conditions were proposed to be developed and optimized ,
- Selection of Mass range,
- Selection of initial separation conditions,
- Nature of the stationary phase,
- Nature of the mobile phase (pH, peak modifier, solvent strength, ratio and flow rate),
- Sensitivity and
- Selection of internal standard.

3. Validation of Bioanalytical method as per Guidelines

The developed method were also proposed to be validated using the various validation parameters such as,

- Accuracy,
- Precision,
- Selectivity,
- Sensitivity
- Linearity and Range,
- Matrix effect,
- Carryover,
- Recovery,
- Dilution Integrity,
- Robustness / ruggedness,
- Stability and
- System suitability.



LITERATURE REVIEW

LITERATURE REVIEW

3.1 Ismail IM et al., (2005) reported metabolism of radiolabelled alosetron was studied in rat, dog, rabbit, mouse and human. The metabolism in rat and dog was studied at a low and an elevated dose designed to generate sufficient quantities of metabolite for definitive identification. A strategy for the characterization of metabolites in cases of extensive metabolism was developed and demonstrated for alosetron. Semi-preparative high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance (NMR) and liquid chromatography-nuclear magnetic resonance (HPLC-NMR) enabled the isolation and characterization of 28 metabolites of alosetron. The characterization of the metabolites in animal excreta facilitated the identification of human systemic metabolites.

3.2 Thomas L. Lloyd et al., (1996) reported method of analysis for the determination of alosetron in human plasma or serum has been developed. The method was fully automated using a laboratory robot in order to improve analytical precision, efficiency and safety. The assay involved solid-phase extraction with reversed-phase HPLC separation and fluorescence detection. A validation exercise over the concentration range of 0.1 to 20 ng/ml demonstrated the selectivity, linearity, sensitivity, accuracy, precision, extraction efficiency, ruggedness and stability of the method. The method has been applied in support of numerous human pharmacokinetic/biopharmaceutic studies over the last five years.

3.3 Wring SA et al., (1994) reported the development of a radioimmunoassay (RIA) for the sub-ng ml-1 determination of alosetron, a potent and selective 5HT3 receptor antagonist, in human urine and saliva is described. The antiserum was raised in Soay sheep following primary and booster immunizations with an immunogen prepared by conjugating alosetron-p-azobenzoic acid to bovine serum albumin (BSA). The radioligand consisted of alosetron specifically 125-iodinated on the 2-position of the imidazole group. The mean (+/- standard deviation) theoretical sensitivity (minimum detectable dose corresponding to the imprecision of the zero standard) of the RIA is 3.2 +/- 2.6 pg ml-1 (n = 12) of alosetron in assay diluent (0.1% m/v gelatine-0.05% m/v sodium azide in 0.1 mol l-1 phosphate buffer solution, pH 7.4). The working calibration range using 0.1 ml samples of saliva and 20-fold diluted urine is 0.10-6.40 ng ml-1 of alosetron. Urine samples were diluted prior to assay to overcome adverse matrix effects; consequently, the lower limit of quantification for undiluted urine is 2.0 ng ml-1 of alosetron. Inter- and intra-assay bias and imprecision over the

working calibration range were generally < +/- 12% and < 13%, respectively, except at the 0.10 ng ml-1 alosetron level, where the corresponding values were < +/- 17.3% and < 20.2%. The antiserum was free from adverse cross-reactivity with either a synthetic precursor of alosetron or with four major metabolites of the drug.

3.4 Lloyd TL et al., (1996) reported a method of analysis for the determination of alosetron in human plasma or serum has been developed. The method was fully automated using a laboratory robot in order to improve analytical precision, efficiency and safety. The assay involved solid-phase extraction with reversed-phase HPLC separation and fluorescence detection. A validation exercise over the concentration range of 0.1 to 20 ng/ml demonstrated the selectivity, linearity, sensitivity, accuracy, precision, extraction efficiency, ruggedness and stability of the method. The method has been applied in support of numerous human pharmacokinetic/biopharmaceutic studies over the last five years.

3.5 Koch K.M et al., (2004) reported to assess the pharmacokinetics of alosetron, its effect on in vivo enzyme activities, and influence of demographic factors during repeated dosing.

Methods: Thirty healthy men and women received 1 mg oral alosetron twice-daily for 29.5 days and a single oral dose of a metabolic probe cocktail before and on the last day of alosetron dosing. Serum alosetron concen-trations were measured on days 1, 8, 15, 22 and 29. Probe-substrate and metabolite concentrations were measured after each cocktail dose.

3.6 Camilleri M, Northcutt AR, Kong S, et al., (2000) Efficacy and safety of alosetron in women with irritable bowel syndrome: a rand-omised, placebo-controlled trial. Lancet 2000; 355: 1035–40.

3.7 Koch KM, Palmer JL, Noordin N, et al., (2002) reported the Sex and age differences in the pharmacokinetics of alosetron. Br J Clin Pharmacol 2002; 53: 238–42.

3.8 Gupta SK, Gooding A.E, Alianti JR et al., (1996) reported the determination of alosetron in human plasma or serum by high-performance liquid chromatography with robotic sample preparation. J Chromatogr B: Biomed Appl 1996; 678: 261–7.

DRUG PROFILE

DRUG PROFILE

4.1 ALOSETRON HYDROCHLORIDE

Chemical IUPAC Name:

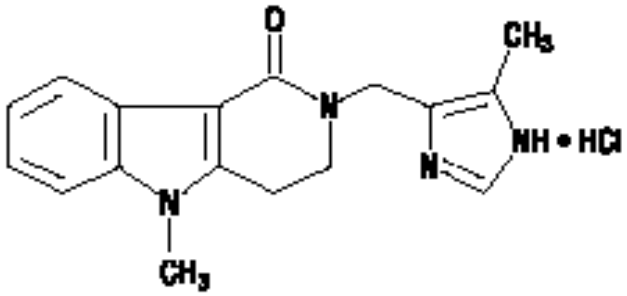
2,3,4,5-tetrahydro-5-methyl-2-[(5-methyl-1H-imidazol-4-yl)methyl]-1H-pyrido[4,3-b]indol-1-one, monohydrochloride.

Empirical formula: $C_{17}H_{18}N_4O•HCl$

Molecular Weight: 330.8 g/mol(salt form)

294.32 g/mol(free form)

Chemical structure:



DESCRIPTION:

ALOSETRON is a potent and selective antagonist of the serotonin 5-HT_3 receptor type. Chemically, alosetron is designated as 2,3,4,5-tetrahydro-5-methyl-2-[(5-methyl-1H-imidazol-4-yl)methyl]-1H-pyrido[4,3-b]indol-1-one, monohydrochloride.

Physio-chemical Properties:

ALOSETRON is a white to beige solid that has a solubility of 61 mg/mL in water, 42 mg/mL in 0.1M hydrochloric acid, 0.3 mg/mL in pH 6 phosphate buffer, and < 0.1 mg/mL in pH 8 phosphate buffer.

pKa value: It has a pKa 13.32.

Protine Binding: 82%

Bioavailability: 50% - 60%

Partition Coefficient: 1.61

Half-life: 1.5-1.7 hours

Mechanism of Action:

Alosetron has an antagonist action on the 5-HT_3 receptors of the enteric nervous system of the gastrointestinal tract. While being a 5-HT_3 antagonist like ondansetron, it is not classified or approved as an antiemetic. Since stimulation of 5-HT_3 receptors is positively correlated with gastrointestinal motility, alosetron's 5-HT_3 antagonism slows the movement of fecal matter through the large intestine, increasing the extent to which water is absorbed, and decreasing the moisture and volume of the remaining waste products.

Pharmacology:

ALOSETRON is a quinolone/fluoroquinolone antibiotic. ALOSETRON is bactericidal and its mode of action depends on blocking of bacterial DNA replication by binding itself to an enzyme called DNA gyrase, which allows the untwisting required to replicate one DNA double helix into two. Notably the drug has 100 times higher affinity for bacterial DNA gyrase than for mammalian. ALOSETRON is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria.

Absorption:

ALOSETRON is rapidly absorbed after oral administration with a mean absolute bioavailability of approximately 50% to 60% (approximate range 30% to > 90%). After administration of radiolabeled alosetron, only 1% of the dose was recovered in the feces as unchanged drug. Following oral administration of a 1-mg alosetron dose to young men, a peak plasma concentration of approximately 5ng/mL occurs at 1 hour. In young women, the mean peak plasma concentration is approximately 9ng/mL, with a similar time to peak.

Side Effects:

Common side effects of ALOSETRON are constipation, abdominal discomfort and pain. Less common side effects of ALOSETRON are nausea, gastrointestinal discomfort and pain, abdominal distention, regurgitation and reflux, hemorrhoids direction.

Indication:

ALOSETRON is indicated only for women with severe diarrhea-predominant irritable bowel syndrome.

Storage:

Keep tightly closed. Store at 2-8 C.

4.2. ALOSETRON D_3 (Internal standard)

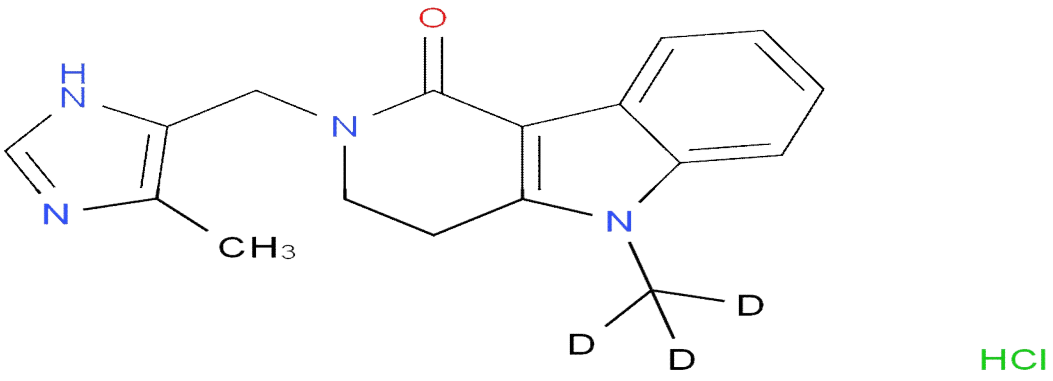
Chemical IUPAC Name:

2, 3, 4, 5-Tetrahydro-5-(methyl-d3)-2-[(4-methyl-1H-imidazol-5-yl)methyl]-1H-pyrido[4,3-b]indol-1-one Hydrochloride

Empirical formula: $C_{17}H_{16}D_3ClN_4O$

Molecular Weight: 331.34 g/mol

Chemical structure:



pKa value: It has a pKa 14.044

MATERIALS AND METHOD

MATERIALS & METHODS

MATERIALS

The method requires the following standards, equipments, reagents and biological matrix.

Note: The standards, equipments, reagents from different manufacturers, with an equivalent specification may be used. Weights, volume mentioned can be scaled up/scaled down based on requirement.

5.1 Analyte Standard

Use authenticated Alosetron reference/working standards for preparation of standard stock solution.

5.1.1 Internal Standard

Use authenticated Alosetron D3 reference/working standards for preparation of internal standard stock solution.

5.2.1 Equipments

Equipment	Make/ Models
LC-MS/MS	Waters TQ MS
UPLC	Waters Acquity UPLC
Precision balances	Mettler Toledo XP205, XP2U
Auto pipettes	Eppendorf
Column	Waters X Bridge BEH Phenyl, 2.5 µm (50 x 2.1 mm)
Vortex mixer	Velp
Plate form Shaker	Heidolph
Centrifuge	Eppendorf
Sonicator	Bandelin sonorex

Deep Freezer (-70^{o}C)	New Brunswick scientific
Deep Freezer (-20^{o}C)	Bio Care
Refrigerator	Samsung

5.2.2 Reagents, Chemicals, Solvents and materials

Reagent/Chemical	Brand	Purity/Grade
Acetonitrile	Fischer Scientific	HPLC
Methanol	J.T Baker	HPLC
Ammonia	Merck	GR
Water	Rankem	HPLC
Volumetric flasks	Borosil	A
Eppendrof tubes	Tarsons	-
RIA vials	Tarsons	-
Tips	Tarsons	-
Multitips	Eppendorf	-
Reagent bottles	Borosil	-

Biological matrix

Use screened interference free human K_2EDTA plasma for preparation of calibration standards and quality control samples.

5.3.1 PREPARATION OF SOLUTIONS

Preparation of Diluent [Acetonitrile: Water (10:90% v/v)]

Transfer 900 mL of Water and 100 mL of Acetonitrile in to 1000 mL reagent bottle and shake well, sonicate and label the solution.

Preparation of Buffer [0.1% v/v Ammonia in water]

Transfer 999 mL of water into 1000 mL reagent bottle and add 1 mL of Ammonia solution. Shake well, sonicate and label the solution.

Preparation of Strong Needle Wash [(Acetonitrile: Water (80:20% v/v)]

Transfer about 800 mL of Acetonitrile and 200 mL of water in to 1000 mL reagent bottle, shake well, sonicate and label the solution.

Preparation of Weak Needle Wash [(Acetonitrile: Water: Ammonia (50:50:0.2% v/v)]

Transfer about 500 mL of Acetonitrile and 500 mL of water in to 1000 mL reagent bottle and add 2 ml of Ammonia solution, shake well, sonicate and label the solution.

Preparation of Seal Wash [(Water: Methanol (95:5% v/v)]

Transfer about 950 mL of water and 50 mL of methanol in to 1000 mL reagent bottle, shake well, sonicate and label the solution.

Preparation of Aqueous standard

Transfer 0.033 mL of spiking solution SS-MQC in to RIA vial and add 0.417 mL of internal standard dilution. Then add 9.550 mL of Diluent. Prepare the aqueous standard as and when required used for system suitability and analytical batch.

Note: As per the requirement, volume of the solutions required can be altered keeping the concentration/composition same.

5.3.2 PREPARATION OF STANDARD SOLUTIONS

Analyte stock solution (w/v) (Alosetron 100.000 µg/mL)

Weigh and transfer about 5.0 mg of Alosetron in to 50 mL volumetric flask. Dissolve with 5 mL of Diluent and make up the volume with the same. Calculate the final concentration by considering its potency, salt and actual amount weighed. Label and store in refrigerator (2ºC to 8ºC). Separate Weighing should be done for Calibration Standards and QC Samples and label the solution.

Internal Standard stock solution (w/v) (Alosetron D3 100.000 µg/mL)

Weigh and transfer about 2.0 mg of Alosetron D3 in to 20 mL volumetric flask. Dissolve with 5mL of acetonitrile and make up the volume with the same. Calculate the final concentration by considering its potency and actual amount weighed. Label as ISA and store in refrigerator (2ºC to 8ºC).

Internal Standard dilution (w/v) (0.050µg/mL ISTD)

Transfer 0.200 mL of internal standard stock in to 2 ml volumetric flask and make up the volume with diluent. And then transfer 0.250 mL into 50 mL volumetric flask and make up the volume with diluent. Label and shall be stored in refrigerator (2ºC to 8ºC).

Preparation of Calibration Standards

Preparation of Calibration Standard spiking solutions for Analyte (Alosetron)

Prepare spiking solutions of calibration standard (CS) from serially diluted solutions using diluent as per the table given below. Store the spiking solutions of CS samples in refrigerator at (2ºC to 8ºC)

Solution ID	Conc. (µg/mL)	Vol. Taken (mL)	Vol. of diluent (mL)	Total Vol.(mL)	Conc. (µg/mL)	CS Spiking Solution ID
ANA	89.2097	1.000	4.000	5.000	17.8419	ANA-IMA01
ANA-IMA01	17.8419	0.400	4.600	5.000	1.4274	SS-08
SS-08	1.4274	4.050	0.950	5.000	1.1562	SS-07
SS-07	1.1562	3.750	1.250	5.000	0.8672	SS-06
SS-06	0.8672	3.380	1.620	5.000	0.5862	SS-05
SS-05	0.5862	2.500	2.500	5.000	0.2931	SS-04
SS-04	0.2931	0.510	4.490	5.000	0.0299	SS-03
SS-03	0.0299	2.500	2.500	5.000	0.0150	SS-02
SS-02	0.0150	2.500	2.500	5.000	0.0075	SS-01

Preparation of Spiked plasma Calibration Standards

Prepare CS by spiking CS spiking solution in screened human K_2EDTA plasma as per the table given below. Label and store in deep freezer (-70± 20 ºC)

Spiking solution ID	Conc. (µg / mL)	Vol. (mL)	Vol. of Matrix. (mL)	Final Vol. (mL)	Final Conc. (ng/ mL)	CS ID
SS-01	0.0075	0.040	1.960	2.000	0.150	CS01
SS-02	0.0150	0.040	1.960	2.000	0.300	CS02
SS-03	0.0299	0.040	1.960	2.000	0.598	CS03
SS-04	0.2931	0.040	1.960	2.000	5.862	CS04
SS-05	0.5862	0.040	1.960	2.000	11.724	CS05
SS-06	0.8672	0.040	1.960	2.000	17.344	CS06
SS-07	1.1562	0.040	1.960	2.000	23.124	CS07
SS-08	1.4274	0.040	1.960	2.000	28.548	CS08

5.4 Preparation of Quality Control Samples

5.4.1 Preparation of QC spiking solutions for Analyte (Alosetron)

Prepare spiking solutions of QC from serially diluted solutions using diluent as per the table given below. Store the spiking solutions of QC samples in refrigerator at (2ºC to 8ºC).

Solution ID	Conc. (µg/mL)	Vol.Taken (mL)	Vol. of diluent (mL)	Total Vol.(mL)	Final Conc. (µg/mL)	QC Spiking Solution ID
ANA	89.2231	1.000	4.000	5.000	17.8446	ANA-IMA01

ANA-IMA01	17.8446	0.580	9.420	10.000	1.0350	SS-HQC
SS-HQC	1.0350	2.350	2.650	5.000	0.4541	SS-MQC
SS-MQC	0.4541	1.800	3.200	5.000	0.1635	SS-IMQC
SS-IMQC	0.1635	0.680	4.320	5.000	0.0222	SS-LQC
SS-LQC	0.0222	1.700	3.300	5.000	0.0076	SS-LLOQC

5.4.2 Preparation of spiked plasma QC samples

Prepare QC standards by spiking QC spiking solution in screened human K_2EDTA plasma as per the table given below. Label and store in deep freezer (-70± 20 ºC).

Spiking solution ID	**Conc. (µg/ mL)**	**Vol. of Spiking Solution (mL)**	**Vol. of Matrix. (mL)**	**Final Vol. (mL)**	**Final Conc. (ng/ mL)**	**QC ID**
SS-LLOQC	0.0076	0.040	1.960	2.000	0.151	LLOQC
SS-LQC	0.0222	0.040	1.960	2.000	0.445	LQC
SS-IMQC	0.1635	0.040	1.960	2.000	3.270	IMQC
SS-MQC	0.4541	0.040	1.960	2.000	9.082	MQC
SS-HQC	1.0350	0.040	1.960	2.000	20.700	HQC

Aliquot approximately 0.300 ml of each CS and QC sample into pre-labeled polypropylene tubes and cap them tightly. Aliquot 0.500 ml of pooled plasma into pre-labeled polypropylene tubes for standard blank and standard zero samples separately. Store CS, QC samples, standard blank and standard zero samples in deep freezer at -70± 20 ºC and -20±5ºC for freeze thaw and Long term stability samples.

5.5 CALIBRATION AND CALCULATIONS

Software used : Mass Lynx Version 4.1

Weighting factor : Linear, $1/X^2$.

Analysis mode : Peak area ratio (Analyte to ISTD) and concentration of Analyte

Calculation : Using following equation by Mass lynx Version 4.1

$y = m x + c$,

Where,

x = Concentration of Analyte in ng/ml

y = Peak area ratio of analyte to ISTD

m = Slope of calibration Curve

c = Intercept on Y- axis

5.6 INSTRUMENTAL PARAMETERS:

5.6.1 METHOD DEVELOPMENT OF ALOSETRON

Method development is a trial and error process. It consists of various steps. Generally it starts with tuning of Alosetron.

5.6.2Tuning of analyte (Alosetron)

Finally stock solution of Alosetron was prepared and diluted to 500ng/ml. This stock dilution was infused in full scan mode. Then from the result, m/z of parent ion was selected. Molecular weight of Alosetron is 294.18 so, m/z peak of 295.18 selected as a parent ion, as we are using positive mode.

Then for fragmentation of parent ion, infused the stock dilution in product ion mode and checked for m/z of various daughter ions obtained. Prominent and suitable daughter ion selected by altering various parameters,

For ex:- Compound dependent parameters:- DP,FP,EP,CE,CXP,CEP

Source dependent parameters:- Nebulizer gas, curtain Gas-2, Temperature

and Ion spray voltage.

It was found that m/z of 295.18 shows a good and stable response. So it selected as daughter ion. Re-optimize the parameters. Finally the parameter selected when the selected daughter ion shows maximum stable response. Then infused stock dilution using m/z of selected parent and daughter ion in Multiple Reaction Monitoring(MRM) mode. Re-optimized the parameters by injecting drug in mobile phase.

5.6.3. TRAIL ON COLUMN AND MOBILE PHASE:

The trails were conducted using different column and by changing the mobile phase composition.

Trail 1

In this trail Ascentis Phenyl column, 3µm (5x4.6mm) and mobile phase of 5mM Ammonium Formate with 0.1% Formic Acid: ACN (30:70) was used.

Trail 2

In this trail Phenomenex Phenyl Hexyl column was used with a Mobile Phase of 5mM Ammonium Acetate with 0.1% Formic Acid: ACN (30:70) was used.

Trail 3

In this trail Ascentis Phenyl column, 3µm (5x4.6mm) was used with a Mobile Phase of

5mM Ammonium Acetate with 0.1% Formic Acid: ACN (30:70) was used.

Conclusion:

In trail **1** peak shape was not good, as it showing tailing. Also the response was less.

In trail **2** peak shape was quite good, as it showing little tailing. Also the response was more as compared with trail 1.

In trail **3** peak shape and response were maximum and stable.

5.6.4. TRIAL ON EXTRACTION PROCEDURE:

In the method Development first step is on extraction techniques. For that different extraction methods were tried as shown below.

BY LIQUID-LIQUID EXTRACTION (LLE)

Trail 01:

Procedure:

Withdraw the spiked plasma samples from the deep freezer and allow them to thaw at room temperature. Aliquot 0.100ml into a clean RIA vial and add 50 µl of Internal Standard (10µg/ml). Vortex well and mix well. Add 2.5ml of TBME and vibramax for 10 minutes. Centrifuge the sample at 4500rpm for 10 minutes at 4ºC. Collect the supernatant of 2.0ml and evaporate till dryness. Reconstitute the residue with 0.500ml of mobile phase and inject 10 µl into LCMS/MS.

Conclusion:

Improper peak shape was observed.

Trail 02:

Procedure:

Withdraw the spiked plasma samples from the deep freezer and allow them to thaw at room temperature. Aliquot 0.100ml into a clean RIA vial and add 50 µl of Internal Standard (10µg/ml). Vortex well and mix well. Add 2.5ml of Dichloromethane: Diethylether (30:70) and vibramax for 10 minutes. Centrifuge the sample at 4500rpm for 10 minutes at 4ºC. Collect the supernatant of 2.0ml and evaporate till dryness. Reconstitute the residue with 0.250ml of mobile phase and inject 10 µl into LCMS/MS.

Conclusion:

Fluctuation in response was observed.

Trail 03:

Procedure:

Withdraw the spiked plasma samples from the deep freezer and allow them to thaw at room temperature. Aliquot 0.100ml into a clean RIA vial and add 50 µl of Internal Standard (10µg/ml).

Vortex well and mix well. Add 2.5ml of Ethyl Acetate and vibramax for 10 minutes. Centrifuge the sample at 4500rpm for 10 minutes at 4ºC. Collect the supernatant of 2.0ml and evaporate till dryness. Reconstitute the residue with 0.250ml of mobile phase and inject 10 µl into LCMS/MS.

Conclusion:

Less response with Irrespective peaks shape was observed.

BY PROTEIN PRECIPITATION EXTRACTION METHOD

Trial 04:

Procedure:

Procedure

Withdraw the spiked plasma samples from the deep freezer and allow them to thaw at room temperature. Aliquot 0.200ml into a clean RIA vial and add 50 µl of Internal Standard (1µg/ml). Vortex well and mix well. Add 0.600ml of Methanol Vibramax for 10 minutes. Centrifuge the sample at 13000rpm for 5 minutes at 4ºC. Add 0.200ml of supernatant solution with 0.400ml of mobile phase and vortex. Inject 10 µl into LCMS/MS.

Conclusion:

Less response with Irrespective peaks shape was observed.

Trail 05:

Procedure

Withdraw the spiked plasma samples from the deep freezer and allow them to thaw at room temperature. Aliquot 0.200ml into a clean RIA vial and add 50 µl of Internal Standard (1µg/ml). Vortex well and mix well. Add 0.600ml of ACN Vibramax for 10 minutes. Centrifuge the sample at 13000rpm for 5 minutes at 4ºC. Add 0.200ml of supernatant solution with 0.400ml of mobile phase and vortex. Inject 10 µl into LCMS/MS.

Conclusion:

Good peak Shape and Constant in response were observed.

5.6.5. PERFORMANCE CHECKING OF SELECTED METHOD

In order to check the performance of selected method, three precision and accuracy batch was processed and evaluated the results for meeting acceptance criteria.

A trail precision and accuracy batch consists of,

-AQS MQC

-RS

-Blank

-Blank along with Internal Standard

-Standards CC1 –CC8

-Six set of QCs

5.7. SAMPLE PROCESSING

1. Ensure that the subdued (yellow) light is on.
2. Retrieve the standard blank, standard zero, CS set and QC samples from the deep freezer and thaw the samples at room temperature.
3. Process all the samples under monochromatic light
4. Homogenize the sample by vortex mixing.
5. Add 50 µL of internal standard (0.050µg/mL Alosetron D3) in to all labelled Eppendorf tubes, except Blank.
6. Transfer 200µL of sample in to the corresponding labelled Eppendorf tubes.
7. Add 600µL of Acetonitrile and vortex.
8. Centrifuge the samples at 13000 rpm at 4°C for 10 minutes.
9. Transfer 0.200 mL of supernatant in to labelled polypropylene RIA vials.
10. Add 0.400 mL of Diluent and vortex the solution.
11. Transfer the samples in to Auto sampler vials.
12. Load the samples into LCMS/MS.

5.8. CHROMATOGRAPHIC CONDITIONS

<table>
<tr><th colspan="2">LC Conditions</th></tr>
<tr><td>Mobile phase & Gradient program</td><td>Pump A: Acetonitrile

Pump B: Buffer (0.1% Ammonia in water)

<table>
<tr><th rowspan="2">Time</th><th rowspan="2">Flow rate (mL/min)</th><th colspan="2">% Ratio</th></tr>
<tr><th>Pump A</th><th>Pump B</th></tr>
<tr><td>Initial</td><td>0.400</td><td>30</td><td>70</td></tr>
<tr><td>0.30</td><td>0.400</td><td>30</td><td>70</td></tr>
<tr><td>0.60</td><td>0.400</td><td>50</td><td>50</td></tr>
<tr><td>0.80</td><td>0.400</td><td>90</td><td>10</td></tr>
<tr><td>1.30</td><td>0.400</td><td>90</td><td>10</td></tr>
<tr><td>1.50</td><td>0.400</td><td>30</td><td>70</td></tr>
<tr><td>2.00</td><td>0.400</td><td>30</td><td>70</td></tr>
</table></td></tr>
<tr><td>Injection volume</td><td>10 µL</td></tr>
<tr><td rowspan="2">Retention time</td><td>Alosetron: 0.80 ± 0.500 min.</td></tr>
<tr><td>ISTD: 0.80 ± 0.500 min.</td></tr>
<tr><td>Column oven temperature</td><td>40 ±5°C</td></tr>
<tr><td>Auto sampler temperature</td><td>5 ± 3°C</td></tr>
<tr><td>Run time</td><td>2.0 min</td></tr>
<tr><td>Strong Needle wash</td><td>Acetonitrile: Water (80:20 % v/v)</td></tr>
<tr><td>Weak Needle wash</td><td>Acetonitrile: Water: Ammonia (50:50:0.2% v/v)</td></tr>
<tr><td>Seal wash</td><td>Methanol: Water (5:95 % v/v)</td></tr>
</table>

Mass Parameters*		
Capillary voltage	0.5 kv	
Desolvation Temperature	550 ^{o}C	
Desolvation Gas Flow	1100 L/hr	
Cone Gas Flow	50 L/hr	
Collision Gas Flow	0.15 mL/min	
Acquisition*		
Parameters	**Alosetron**	**ISTD**
Transition	295.13/201.09 (m/z)	298.10/204.07 (m/z)
Polarity	Positive	Positive
MS1 resolution	Unit	Unit
MS2 resolution	Unit	Unit
Dwell time (sec)	0.100	0.100
Cone Voltage	24	24
Collision energy	16	16

Instrumentation

Detector	Waters XEVO TQ Triple Quad LC/MS
Ion source	ESI+
Pump	Waters Acquity Binary Solvent Manager
Auto sampler	Waters Acquity Sample Manager
Column oven	Waters Acquity
Column	Waters X Bridge BEH Phenyl, 2.5 µm (50 x 2.1 mm)

*Mass spectrometer parameters may differ slightly between instruments

Sample Processing Check List

MV No: **Batch ID:**

Date:

S.No.	Procedure	Y/N	Initial
1	Ensure that the subdued (yellow) light is on.		
2	Retrieve the standard blank, Standard zero, CS set, QC samples from the deep freezer. Process all the samples under monochromatic light.		
3	Thaw the samples at room temperature.		
4	Homogenize the sample by vortex mixing.		
5	Add 50 µL of internal standard (0.050µg/mL Alosetron D3) in to all labelled Eppendorf tubes, except Blank.		
6	Transfer 200 µL of sample in to the corresponding labelled Eppendorf tubes.		
7	Add 600 µL of Acetonitrile and vortex.		
8	Centrifuge the samples at 13000 rpm at 4°C for 10 minutes		
9	Transfer 0.200 mL of supernatant in to labelled polypropylene RIA vials.		
10	Add 0.400 mL of Diluent and vortex the solution.		
11	Transfer the samples in to Auto sampler vials.		
12	Load the samples into LCMS/MS.		

Remarks: **Verified by:**

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

6.1 Chromatography

A typical chromatogram obtained from a processed blank human K_2EDTA plasma sample is illustrated in Figure 1. Representative chromatograms of the lower limit of quality control, low, intermediate, medium and high quality control (QC) and upper limit of quantification samples are displayed in Figures 1, 2, 3,4,5,6 & 7 respectively.

The retention times of Alosetron and internal standard are approximately 0.80 and 0.79 minutes, respectively. The overall chromatography time is 2 minutes.

6.2 Selectivity

Selectivity was evaluated by analyzing thirteen different human K_2EDTA plasma lots (eleven normal lots and one Haemolysed and one Lipemic lot) obtained from thirteen independent sources. No significant interference observed at the retention time of analyte and internal standard for 12 and 13 lots respectively. Results are presented in Table 1.

Table 1: Selectivity

S. No.	Blank Plasma Lot ID	Extracted blank		LLOQ		% Interference	
		Area at the RT of Analyte	Area at the RT of IS	Analyte Area	IS Area	Area at the RT of Analyte	Area at the RT of IS
1	MT-113/13_BLK	92	75	1411	66602	6.520	0.113
2	MT-142/13_BLK	52	24	1328	66627	3.916	0.036
3	MT-147/13_BLK	52	14	1311	68625	3.966	0.020
4	MT-150/13_BLK	0	0	1278	67045	0.000	0.000
5	MT-151/13_BLK	62	11	1385	68661	4.477	0.016

6	MT-152/13_BLK	10	10	735	34134	1.361	0.029
7	MT-154/13_BLK	2356	11	1288	67850	182.919	0.016
8	MT-155/13_BLK	28	0	1207	64490	2.320	0.000
9	MT-156/13_BLK	26	0	1163	63031	2.236	0.000
10	MT-157/13_BLK	13	0	1316	65091	0.988	0.000
11	MT-158/13_BLK	14	0	1144	62285	1.224	0.000
12	MT-153/13(H)_BLK	44	0	1094	61827	4.022	0.000
13	MT-005/12(L)_BLK	125	12	1120	63751	11.161	0.019

6.3 Matrix Effect

Blank plasma samples of thirteen different human K_2EDTA plasma (eleven normal lots and one Haemolysed and one Lipemic lot) sources were processed and spiked with aqueous low quality control and high quality control (post extraction addition) and analyzed in a single run along with diluted pure standard at each concentration level.

Table 2: Matrix Effect

QC ID	Aqueous Analyte Area Ratio	Aqueous IS Area ratio	Blank Plasma Lot ID	Post Extracted LQC with IS		Matrix Effect		
				Analyte Area Ratio	IS Area Ratio	ME of Analyte	ME of IS	IS-normalized MF
LQC	0.048	20.872	LQC_MT-113/13	0.050	20.076	1.042	0.972	1.072
	0.047	21.074	LQC_MT-142/13	0.049	20.331	1.021	0.984	1.037
	0.048	20.676	LQC_MT-147/13	0.048	20.641	1.000	0.999	1.001
	0.048	20.793	LQC_MT-150/13	0.049	20.385	1.021	0.987	1.035
	0.049	20.258	LQC_MT-151/13	0.049	20.538	1.021	0.994	1.027
	0.049	20.281	LQC_MT-152/13	0.052	19.250	1.083	0.932	1.163
			LQC_MT-154/13	0.050	20.111	1.042	0.973	1.070
			LQC_MT-155/13	0.050	20.110	1.042	0.973	1.070

			LQC_MT-156/13	0.050	20.000	1.042	0.968	1.076
			LQC_MT-157/13	0.048	20.821	1.000	1.008	0.992
			LQC_MT-158/13	0.050	20.039	1.042	0.970	1.074
			LQC_MT-153/13(H)	0.048	20.663	1.000	1.000	1.000
			LQC_MT-005/12(L)	0.048	20.634	1.000	0.999	1.001
AVERAGE	0.048	20.659			AVERAGE	1.027	0.982	1.047
SD	0.001	0.328			SD	0.025	0.020	0.047
% CV	2.08	1.59			% CV	2.43	2.04	4.49
	2.405	0.416	HQC_MT-113/13	2.391	0.418	0.992	1.008	0.984
	2.407	0.415	HQC_MT-142/13	2.411	0.415	1.000	0.999	1.001
	2.418	0.414	HQC_MT-147/13	2.383	0.420	0.989	1.011	0.978
	2.429	0.412	HQC_MT-150/13	2.413	0.414	1.001	0.999	1.003
	2.403	0.416	HQC_MT-151/13	2.428	0.412	1.007	0.993	1.015
	2.395	0.418	HQC_MT-152/13	2.407	0.415	0.999	1.001	0.998
HQC			HQC_MT-154/13	2.429	0.412	1.008	0.992	1.016
			HQC_MT-155/13	2.396	0.417	0.994	1.006	0.989
			HQC_MT-156/13	2.415	0.414	1.002	0.998	1.004
			HQC_MT-157/13	2.429	0.412	1.008	0.992	1.016
			HQC_MT-158/13	2.537	0.394	1.053	0.950	1.109
			HQC_MT-153/13(H)	2.413	0.414	1.001	0.999	1.003
			LQC_MT-005/12(L)	2.404	0.416	0.998	1.002	0.995
AVERAGE	0.615	1.628			AVERAGE	1.004	0.996	1.008
SD	0.004	0.010			SD	0.016	0.015	0.032
% CV	0.65	0.61			% CV	1.59	1.51	3.18

H- Haemolysed, L- Lipemic

The percentage CV of matrix effect for analyte was found to be 2.43 and 1.59 for low and high quality control samples respectively. The percentage CV of IS normalized matrix factor was found to be 4.49 and 3.18 for low and high quality control samples respectively. Results are presented in Table 2.

6.4 Carry Over Test

Auto sampler carry over test was performed by injecting an extracted ULOQ standard followed by an extracted blank and found that there is no significant carry over in all two instruments tested (BA-MS-07 and BA-MS-08). Results were presented in Table 3.

Table 3: Carryover Test

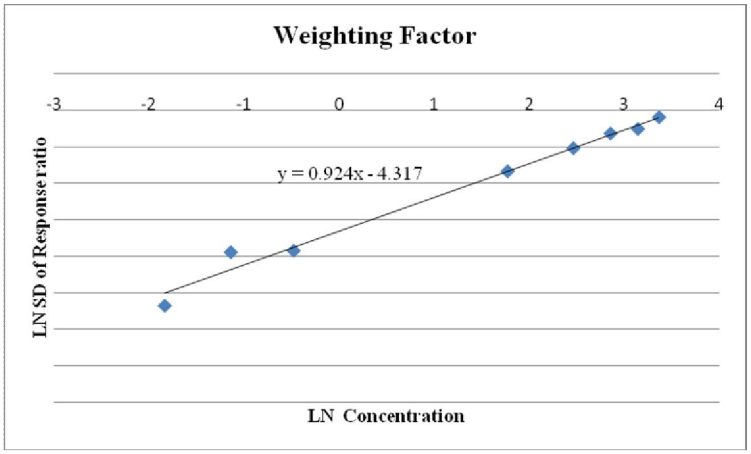
	Instrument ID: BA-MS-07		**Instrument ID: BA-MS-08**	
SAMPLE	**Area at the retention of Analyte**	**Area at the retention of IS**	**Area at the retention of Analyte**	**Area at the retention of IS**
Extracted Blank	18	0	94	24
Extracted ULOQ with IS	257577	87549	141321	48418
Reinjection of Extracted Blank	38	11	115	22
Extracted LLOQ with IS	1520	89826	757	45737
% CARRY OVER	**1.32**	**0.01**	**2.77**	**0.00**

6.5 Weighting Factor of Regression Method

To determine whether to fit the data for the calibration curves by weighted or unweight linear regression, the functional dependence of the natural logarithm of standard deviation of the analyte/internal standard area ratio on natural logarithm of sample concentration was evaluated. The individual data used for the determination of the power of weights for human K_2 EDTA plasma calibration curves are presented, in Table 4. The weighting factor to be used is $1/X^2$ since the slope (m) of the regression line equals 0.924.

Table 4: Weighting Factor of Regression Method

	Analyte / Internal Standard Response Ratio							
CS ID	**CS-1**	**CS-2**	**CS-3**	**CS-4**	**CS-5**	**CS-6**	**CS-7**	**CS-8**
Nominal Conc.	**0.160**	**0.320**	**0.620**	**5.880**	**11.740**	**17.360**	**23.140**	**28.920**
PA 1	0.017	0.041	0.066	0.602	1.18	1.749	2.299	2.92
PA 2	0.017	0.033	0.065	0.599	1.188	1.786	2.291	2.842
PA 4	0.014	0.026	0.052	0.481	0.959	1.432	1.913	2.359
SD	**0.002**	**0.008**	**0.008**	**0.069**	**0.130**	**0.195**	**0.221**	**0.304**
Ln (SD)	**-6.3584**	**-4.8921**	**-4.8523**	**-2.67351**	**-2.04049**	**-1.6369**	**-1.51148**	**-1.19109**
Ln(Conc)	**-1.8326**	**-1.1394**	**-0.478**	**1.77156**	**2.463**	**2.85417**	**3.14156**	**3.36453**



Slope m= 0.924

Acceptance Criteria:

Un weighted- $m < 0.25$

$1/X - 0.25 < m < 0.75$

$I/X^2 - m > 0.75$

6.6 Calibration Curves

Linearity was evaluated using eight different concentrations. Calibration curves were found to be consistently accurate and precise over the 0.160 to 28.920 ng/mL calibration range. The coefficient of determination (r^2) is greater than or equal to 0.9991. Back-calculations were made from the calibration curves to determine Alosetron concentrations of each calibration standard. Data are presented in Tables 5 and 6. A typical calibration curve is presented in Figure 7.

Table 5: Summary of Calibration Curve Parameters

Y = mx+c ($1/x^2$ weighted)			
Batch ID	**Slope**	**Intercept**	**r^2**
PA 01	0.1013	0.0007	0.9996
PA 02	0.1007	0.0013	0.9996
PA 04	0.0821	0.0003	0.9997
RUG	0.1038	0.0013	0.9991
PBPA	0.0790	0.0005	0.9999

Table 6: Back-Calculated Standards from Each Calibration Curve

Calibration Standard ID		**CS 01**	**CS 02**	**CS 03**	**CS 04**	**CS 05**	**CS 06**	**CS 07**	**CS 08**
Nominal Concentration (ng/mL)		**0.160**	**0.320**	**0.620**	**5.880**	**11.740**	**17.360**	**23.140**	**28.920**
Back Calculated Concentration (ng/ml)	**PA 01**	0.158	R	0.645	5.930	11.639	17.249	22.681	28.804
	PA 02	0.160	0.315	0.632	5.938	11.786	17.728	22.750	28.228
	PA 04	0.162	0.310	0.635	5.854	11.685	17.447	23.316	28.743
	RUG	0.156	0.329	0.650	5.816	11.651	17.559	22.580	28.362
	PBPA	0.161	0.314	0.629	5.879	11.808	17.372	23.079	28.777
AVERAGE		**0.159**	**0.317**	**0.638**	**5.883**	**11.714**	**17.471**	**22.881**	**28.583**
SD		**0.002**	**0.008**	**0.009**	**0.051**	**0.078**	**0.183**	**0.307**	**0.268**
% CV		**1.26**	**2.52**	**1.41**	**0.87**	**0.67**	**1.05**	**1.34**	**0.94**
% ACCURACY		**99.38**	**99.06**	**102.90**	**100.05**	**99.78**	**100.64**	**98.88**	**98.83**

6.7 Between-Run Accuracy and Precision

The between-run accuracy and precision evaluation were assessed by the repeated analysis of human K_2EDTA plasma samples containing different concentrations of Alosetron on separate occasions. A single run consisted of a calibration curve plus 6 replicates of lower limit of quantification, low, intermediate, medium and high quality control samples.

The between-run precision in terms of coefficients of variation ranged between 0.66 and 10.98 %. The between-run accuracy ranged between 98.58 and 108.13 %. Results are presented in Table 7.

Table 7: Between-Run Accuracy and Precision

QC ID		LLOQC	LQC	IMQC	MQC	HQC
Nominal Concentration (ng/ml)		**0.160**	**0.460**	**3.180**	**9.100**	**22.760**
Back Calculated Concentration (ng/ml)	PA01	0.178	0.482	3.179	9.074	22.458
		0.186	0.476	3.372	9.054	22.474
		0.161	0.481	3.150	9.017	22.451
		0.206	0.485	3.210	9.123	22.598
		0.229	0.465	3.219	9.078	22.370
		0.175	0.475	3.231	9.114	22.543
	PA02	0.159	0.480	3.136	9.034	22.554
		0.181	0.473	3.184	8.886	22.689
		0.162	0.471	3.161	9.682	22.582
		0.168	0.478	3.130	9.044	22.419
		0.163	0.475	3.161	9.004	22.262
		0.154	0.459	3.144	9.001	22.455
	PA04	0.160	0.462	3.192	9.034	22.205
		0.160	0.456	3.101	9.072	22.199
		0.166	0.471	3.191	9.129	22.509
		0.164	0.475	3.154	9.038	22.350
		0.171	0.486	3.113	8.859	22.546
		0.177	0.455	3.133	8.991	22.199
AVERAGE		**0.173**	**0.473**	**3.176**	**9.069**	**22.437**
SD		**0.019**	**0.010**	**0.061**	**0.169**	**0.147**

%CV	**10.98**	**2.11**	**1.92**	**1.86**	**0.66**
% ACCURACY	**108.13**	**102.83**	**99.87**	**99.66**	**98.58**

6.8 Within-Run Accuracy and Precision

Within-run accuracy and precision evaluations were performed by analyzing replicate concentrations of Alosetron in human K_2EDTA plasma. The run consisted of a calibration curve plus a total of 6 replicates of each of the LLOQC, lower, intermediate, medium and higher quality control samples.

The within-run precision in terms of coefficients of variation ranged between 0.35 and 12.70 %. The within-run accuracy ranged between 98.13 and 118.13 %. Results are presented in Table 8.

Table 8: Within-Run Accuracy and Precision

QC ID		**LLOQC**	**LQC**	**IMQC**	**MQC**	**HQC**
Nominal Concentration (ng/ml)		**0.160**	**0.460**	**3.180**	**9.100**	**22.760**
Back Calculated Concentration (ng/ml)	PA01	0.178	0.482	3.179	9.074	22.458
		0.186	0.476	3.372	9.054	22.474
		0.161	0.481	3.150	9.017	22.451
		0.206	0.485	3.210	9.123	22.598
		0.229	0.465	3.219	9.078	22.370
		0.175	0.475	3.231	9.114	22.543
AVERAGE		**0.189**	**0.477**	**3.227**	**9.077**	**22.482**
SD		**0.024**	**0.007**	**0.077**	**0.039**	**0.079**
%CV		**12.70**	**1.47**	**2.39**	**0.43**	**0.35**
% ACCURACY		**118.13**	**103.70**	**101.48**	**99.75**	**98.78**
QC ID		**LLOQC**	**LQC**	**IMQC**	**MQC**	**HQC**

Nominal Concentration (ng/ml)		**0.160**	**0.460**	**3.180**	**9.100**	**22.760**
Back Calculated Concentration (ng/ml)	PA02	0.159	0.480	3.136	9.034	22.554
		0.181	0.473	3.184	8.886	22.689
		0.162	0.471	3.161	9.682	22.582
		0.168	0.478	3.130	9.044	22.419
		0.163	0.475	3.161	9.004	22.262
		0.154	0.459	3.144	9.001	22.455
AVERAGE		**0.165**	**0.473**	**3.153**	**9.109**	**22.494**
SD		**0.009**	**0.007**	**0.020**	**0.287**	**0.149**
%CV		**5.45**	**1.48**	**0.63**	**3.15**	**0.66**
% ACCURACY		**103.13**	**102.83**	**99.15**	**100.10**	**98.83**
QC ID		**LLOQC**	**LQC**	**IMQC**	**MQC**	**HQC**
Nominal Concentration (ng/ml)		**0.160**	**0.460**	**3.180**	**9.100**	**22.760**
Back Calculated Concentration (ng/ml)	PA04	0.160	0.462	3.192	9.034	22.205
		0.160	0.456	3.101	9.072	22.199
		0.166	0.471	3.191	9.129	22.509
		0.164	0.475	3.154	9.038	22.350
		0.171	0.486	3.113	8.859	22.546
		0.177	0.455	3.133	8.991	22.199
AVERAGE		**0.166**	**0.468**	**3.147**	**9.021**	**22.335**
SD		**0.007**	**0.012**	**0.039**	**0.092**	**0.161**
%CV		**4.22**	**2.56**	**1.24**	**1.02**	**0.72**
% ACCURACY		**103.75**	**101.74**	**98.96**	**99.13**	**98.13**

6.9 Ruggedness

The ruggedness of the method was assessed by analyzing a precision and accuracy batch using a different serial number of column (Serial No.:01113126616013, BA-CL-100) by different analyst in different instrument of similar configuration (Instrument ID.: BA-MS-08).

The precision in terms of coefficients of variation ranged between 0.53 and 2.91 %. The accuracy percentages of nominal concentrations ranged between 99.70 and 107.50 %. Results are presented in Table 9.

Table 9: Ruggedness

Instrument ID : BA-MS-08

Column ID : BA-CL-100

Batch ID : RUG

QC ID	LLOQC	LQC	IMQC	MQC	HQC
Nominal Concentration (ng/ml):	**0.160**	**0.460**	**3.180**	**9.100**	**22.760**
Back Calculated Concentration of QC samples (ng/ml) :	0.180	0.471	3.185	9.095	22.744
	0.176	0.471	3.191	9.322	22.496
	0.171	0.475	3.181	8.927	22.766
	0.171	0.482	3.180	9.121	22.844
	0.169	0.471	3.195	8.979	22.774
	0.167	0.456	3.146	8.992	22.567
Mean :	**0.172**	**0.471**	**3.180**	**9.073**	**22.699**
SD :	**0.005**	**0.009**	**0.017**	**0.142**	**0.135**
%CV :	**2.91**	**1.91**	**0.53**	**1.57**	**0.59**
%ACCURACY	**107.50**	**102.39**	**100.00**	**99.70**	**99.73**

6.10 Production Batch Precision and Accuracy

A total number of 145 samples were processed and analyzed in a single batch to simulate the production batch; the precision and accuracy of the quality control samples were calculated, the precision in terms of coefficients of variation ranged between 1.36 and 3.72 %. The accuracy ranged between 99.19 and 105.22 %. Results are presented in Table 10.

Table 10: Production Batch Precision and Accuracy

QC ID	LQC	IMQC	MQC	HQC
Nominal Concentration (ng/ml):	**0.460**	**3.180**	**9.100**	**22.760**
Back Calculated QC concentrations	0.466	3.245	9.152	22.749
	0.500	3.213	9.077	22.776
	0.536	3.239	9.159	22.693
	0.477	3.375	9.118	22.588
	0.479	3.197	9.152	22.474
	0.469	3.221	9.167	22.437
	0.469	3.237	9.078	22.357
	0.494	3.197	9.109	23.155
	0.475	3.185	9.577	22.378
	0.470	3.183	9.086	22.923
	0.456	3.204	9.126	22.158
	0.476	3.332	8.891	22.077
	0.479	3.254	9.140	22.539
	0.498	3.154	9.061	22.870

	0.521	3.310	9.237	22.739
	0.476	3.412	9.149	22.482
	0.490	3.251	9.153	22.406
	0.474	3.197	9.170	22.457
	0.488	3.217	9.148	22.407
	0.488	3.213	9.174	23.283
	0.455	3.199	9.517	22.302
	0.473	3.200	9.099	23.035
	0.475	3.192	9.142	22.077
	0.481	3.164	8.979	21.988
	0.473	3.242	9.170	22.735
	0.496	3.178	9.078	22.905
	0.529	3.248	9.174	22.720
	0.470	3.411	9.184	22.626
	0.480	3.235	9.096	22.287
	0.488	3.212	9.172	22.433
	0.492	3.224	9.029	22.222
	0.481	3.203	9.154	23.118
Mean :	**0.484**	**3.236**	**9.147**	**22.575**
SD :	**0.018**	**0.065**	**0.124**	**0.328**
%CV :	**3.72**	**2.01**	**1.36**	**1.45**
%ACCURACY	**105.22**	**101.76**	**100.52**	**99.19**

6.11 Recovery

6.11.1. Recovery of Analyte

Recovery of Alosetron was evaluated by comparing mean analyte responses of six processed samples of low, medium and high quality control samples to mean analyte responses of diluted pure standard solutions. The mean recovery values were found to be 77.36%, 74.39% and 75.37% at low, medium and high quality control levels, respectively. The global mean recovery was found to be 75.71% with the coefficient of variation 2.00. Results are presented in Table 11.

Table 11: Recovery of Analyte

S. No.	LQC		MQC		HQC	
	0.460 ng/mL		9.100 ng/mL		22.760 ng/mL	
	Aqueous Analyte response	Extracted Analyte response	Aqueous Analyte response	Extracted Analyte response	Aqueous Analyte response	Extracted Analyte response
1	5370	4533	115560	86882	282397	210493
2	5837	4537	116186	83934	282548	213754
3	6021	4495	116627	91779	282603	212538
4	5907	4510	115299	86300	282496	213983
5	5960	4553	115520	84556	285429	212263
6	5952	4483	116660	84194	279776	214760
Mean	**5841.167**	**4518.500**	**115975.333**	**86274.167**	**282541.500**	**212965.167**
SD (±)	**238.786**	**26.935**	**595.831**	**2947.164**	**1789.549**	**1527.533**
CV (%)	**4.09**	**0.60**	**0.51**	**3.42**	**0.63**	**0.72**
% Recovery	**77.36**		**74.39**		**75.37**	
Mean Recovery	**75.71**					
% CV	**2.00**					

6.11.2. Recovery of Internal standard

Recovery of internal standard, mean internal standard responses of eighteen processed samples were compared with mean internal standard responses of eighteen diluted pure internal standard injections. Mean recovery value for the internal standard was found to be 79.34%. Results are presented in Table 12.

Table 12: Recovery of Internal Standard

	Aqueous Area	Extracted Area
	107839	91363
	118455	92868
	119980	92380
	119089	91356
	118488	92718
	119814	94470
	117514	95423
	118062	93713
	118745	94060
	117858	94675
	117085	93177
	117892	92811
	118015	92674
	118714	93550
	118347	93460

	118243	94780
	119613	94680
	117739	94969
AVERAGE	**117861**	**93507**
SD	**2620.755**	**1194.610**
% CV	**2.22**	**1.28**
% Recovery of IS	**79.34**	

6.12 Sensitivity (Lower Limit of Quantification)

The sensitivity of the assay was determined by calculating the signal-to-noise (S/N) ratio of the chromatographic peak of the lowest calibration standard in each validation run containing a calibration curve. Sensitivity was considered sufficient if the S/N ratio is at least 5.

The lower limit of quantification, i.e. the lowest standard is 0.160 ng/mL, the coefficient of variation found to be 1.26 % and the accuracy is 99.38 %. Results were presented in Table 13.

Table 13: Sensitivity (Lower Limit of Quantification)

Nominal conc.: 0.160 ng/mL		Calculated Conc. (ng/mL)	S/N Ratio
PA BATCH ID	**PA01**	0.158	394.562
	PA02	0.160	312.576
	RUG	0.156	1190.031
	PA04	0.162	184.602
	PBPA	0.161	680.612
	Mean	**0.159**	
	SD	**0.002**	
	% CV	**1.26**	
	% Accuracy	**99.38**	

6.13 Dilution Integrity

Six replicates of dilution quality control samples (DQC) were respectively diluted two ($1/2^{th}$ dilution) and four times ($1/4^{th}$ dilution) in human K_2EDTA plasma prior to sample processing and analysis. The calculated concentrations, including the dilution factor, yielded coefficients of variation of 1.12 and 0.48%, for 1/2th and 1/4th, respectively. Percentages of nominal concentrations are 99.53 and 98.81%, respectively. Results are presented in Table 14.

Table 14: Dilution Integrity

QC ID	DQC	
	DQC Concentration: ng/mL	
	2T	4T
Concentration (ng/ml)	**45.540**	**45.540**
Back Calculated Concentration (ng/ml)	44.774	45.347
	44.749	44.706
	45.891	45.029
	45.895	45.071
	45.407	44.857
	45.232	44.963
AVERAGE	**45.325**	**44.996**
SD	**0.509**	**0.217**
% CV	**1.12**	**0.48**
% ACCURACY	**99.53**	**98.81**

6.14 Reinjection Reproducibility

Six replicates of LQC and HQC samples from PA02 batch were re-injected after 15 hours 21 minutes along with calibration standards, back calculated concentration of QC samples were calculated using original calibration standards and re-injected calibration standards, accuracy and precision of QC samples were calculated and reported. Results were presented in Table 15.

The result shows that the results are reproducible when re-injected the batch as a whole and the samples individually.

Table 15: Reinjection Reproducibility

PA Batch ID : PA 02

QC ID	Back Calculated Concentration with re-injected Calibration Curve (ng/ml)		Back Calculated Concentration with original Calibration Curve (ng/ml)	
	LQC	**HQC**	**LQC**	**HQC**
Nominal Concentration (ng/ml)	**0.460**	**22.760**	**0.460**	**22.760**
	0.477	22.379	0.480	22.554
	0.464	22.640	0.473	22.689
	0.462	22.669	0.471	22.582
	0.475	22.523	0.478	22.419
	0.471	22.145	0.475	22.262
	0.452	22.450	0.459	22.455
AVERAGE	**0.467**	**22.468**	**0.473**	**22.494**
SD	**0.009**	**0.210**	**0.007**	**0.163**
%CV	**1.93**	**0.93**	**1.48**	**0.72**
% ACCURACY	**101.52**	**98.72**	**102.83**	**98.83**

6.15 Stability

6.15.1. Auto sampler stability

Six replicates of Low (LQC) and high (HQC) Quality control samples of Stability-01 Batch were retained after sample analysis and kept stored in auto sampler at 5ºC for 21 hours 40 minutes. Stability samples were analyzed in a single run with freshly spiked calibration curve and quality control samples (Comparison Samples- time zero). Concentrations were calculated to determine % stability when compared to time zero. Alosetron is found to be stable for 21 hours 40 minutes at 5ºC in auto sampler with % stability of 99.79 and 100.14% for LQC and HQC respectively. Results are presented in Table 16.

Table 16: Auto Sampler Stability

Stability Duration : 21 hours 40 minutes at 5ºC

QC ID	**Comparison Sample**		**Stability Sample**	
	LQC	**HQC**	**LQC**	**HQC**
Nominal Concentration (ng/ml)	**0.460**	**22.760**	**0.460**	**22.760**
Back Calculated Concentration (ng/ml)	0.485	22.284	0.462	22.059
	0.478	22.075	0.471	22.263
	0.458	23.278	0.469	23.36
	0.476	22.981	0.466	22.92
	0.495	23.42	0.473	22.287
	0.452	22.19	0.498	23.529
AVERAGE	**0.474**	**22.705**	**0.473**	**22.736**
SD	**0.016**	**0.592**	**0.013**	**0.622**
%CV	**3.38**	**2.61**	**2.75**	**2.74**
% ACCURACY	**103.04**	**99.76**		
% STABILITY			**99.79**	**100.14**

6.15.2. Bench Top Stability (Analyte in Matrix at Room Temperature)

Six replicates of low and high quality control samples were processed after keeping the samples at room temperature for approximately 16 hours 25 minutes (Stability Samples) and analyzed with freshly spiked calibration curve and quality control samples (Comparison Samples). Concentrations were calculated to determine % stability over time. Alosetron is found to be stable in human K_2EDTA plasma for 16 hours 25 minutes at room temperature with % stability of 101.95 and 101.05% for LQC and HQC respectively. Results are presented in Table 17.

Table 17: Bench Top Stability (Analyte in Matrix at Room Temperature)

Stability Duration : 16 hours 25 minutes

QC ID	**Comparison Sample**		**Stability Sample**	
	LQC	**HQC**	**LQC**	**HQC**
Nominal Concentration (ng/ml)	**0.460**	**22.760**	**0.460**	**22.760**
Back Calculated Concentration (ng/ml)	0.467	21.978	0.475	22.119
	0.469	21.885	0.472	22.443
	0.449	22.438	0.457	22.692
	0.471	22.076	0.489	22.361
	0.478	21.942	0.458	22.403
	0.436	22.245	0.476	21.943
AVERAGE	**0.462**	**22.094**	**0.471**	**22.327**
SD	**0.016**	**0.211**	**0.012**	**0.262**
%CV	**3.46**	**0.96**	**2.55**	**1.17**
% ACCURACY	**100.43**	**97.07**		
% STABILITY			**101.95**	**101.05**

6.15.3. Wet Extract Stability at Room Temperature

Six replicates of low and high quality control samples were processed and reconstituted with diluent. The samples were transferred into injector vials and kept for 25 hours 45 minutes on bench at room temperature. Samples were injected after 25 hours 45 minutes and analyzed with freshly spiked calibration curve and quality control samples (Comparison Samples). Concentrations were calculated to determine % stability. Processed samples of Alosetron are found to be stable for 25 hours 45 minutes at room temperature with a % stability of 91.06 and 96.48% for LQC and HQC respectively. Results are presented in Table 18.

Table 18: Wet extract stability at Room Temperature

Stability Duration : 25 hours 45 minutes

QC ID	**Comparison Sample**		**Stability Sample**	
	LQC	**HQC**	**LQC**	**HQC**
Nominal Concentration (ng/ml)	**0.460**	**22.760**	**0.460**	**22.760**
Back Calculated Concentration (ng/ml)	0.465	23.485	0.403	22.243
	0.501	23.453	0.462	21.992
	0.479	23.472	0.478	22.124
	0.499	23.618	0.444	22.480
	0.500	23.376	0.444	22.687
	0.508	22.249	0.456	23.214
AVERAGE	**0.492**	**23.276**	**0.448**	**22.457**
SD	**0.016**	**0.509**	**0.025**	**0.447**
%CV	**3.25**	**2.19**	**5.58**	**1.99**
% ACCURACY	**106.96**	**102.27**		
% STABILITY			**91.06**	**96.48**

6.15.4. Wet Extract Stability at Refrigerator (2-8 ºC)

Six replicates of low and high quality control samples were processed and reconstituted with diluent. The samples were transferred into injector vials and kept for 46 hours 44 minutes in refrigerator. Samples were injected after 46 hours 44 minutes and analyzed with freshly spiked calibration curve and quality control samples (Comparison Samples). Concentrations were calculated to determine % stability. Processed samples of Alosetron are found to be stable for 46 hours 44 minutes in refrigerated condition with% stability of 95.93 and 95.88% for LQC and HQC respectively. Results are presented in Table 19.

Table 19: Wet Extract Stability in Refrigerator

Stability Duration : 46 hours 44 minutes

QC ID	**Comparison Sample**		**Stability Sample**	
	LQC	**HQC**	**LQC**	**HQC**
Nominal Concentration (ng/ml)	**0.460**	**22.760**	**0.460**	**22.760**
Back Calculated Concentration (ng/ml)	0.465	23.485	0.476	22.657
	0.501	23.453	0.475	22.295
	0.479	23.472	0.478	22.346
	0.499	23.618	0.476	22.208
	0.500	23.376	0.472	22.068
	0.508	22.249	0.453	22.320
AVERAGE	**0.492**	**23.276**	**0.472**	**22.316**
SD	**0.016**	**0.509**	**0.009**	**0.195**
%CV	**3.25**	**2.19**	**1.91**	**0.87**
% ACCURACY	**106.96**	**102.27**		
% STABILITY			**95.93**	**95.88**

6.15.5. Freeze Thaw Stability at -20°C and -70°C

Low and high quality control samples were prepared, aliquoted and frozen at -20 ± 5°C and -70 ± 20°C. Six replicates of LQC and HQC samples were processed after four freeze thaw cycle in both the temperature (stability samples). Freeze-Thaw samples were analyzed with freshly spiked calibration curve and quality control samples (Comparison Samples) in a single run.

Alosetron samples stored at -20 ± 5°C were found to be stable in human K_2EDTA plasma after four freeze-thaw cycles with % stability of 99.79 and 100.50% for LQC and HQC respectively.

Alosetron samples stored at -70 ± 20°C were found to be stable in human K_2EDTA plasma after four freeze-thaw cycles with % stability of 99.79 and 100.54 for LQC and HQC respectively. Results are presented in Table 20.

Table 20: Freeze Thaw Stability at -70°C and -20°C

Number of cycles : Four

QC ID	**Comparison Sample**		**Stability Sample**		**Stability Sample**	
			-70 ± 20°C		-20 ± 5°C	
	LQC	**HQC**	**LQC**	**HQC**	**LQC**	**HQC**
Nominal Concentration (ng/ml)	**0.460**	**22.760**	**0.460**	**22.760**	**0.460**	**22.760**
Back Calculated Concentration (ng/ml)	0.480	22.231	0.456	22.199	0.478	22.191
	0.479	22.112	0.482	22.051	0.482	22.681
	0.460	22.699	0.460	22.709	0.468	22.130
	0.486	22.274	0.478	22.649	0.456	22.678
	0.493	22.118	0.485	22.444	0.494	22.390
	0.450	22.454	0.480	22.555	0.464	22.494
AVERAGE	**0.475**	**22.315**	**0.474**	**22.435**	**0.474**	**22.427**
SD	**0.016**	**0.226**	**0.012**	**0.260**	**0.014**	**0.235**

% CV	**3.37**	**1.01**	**2.53**	**1.16**	**2.95**	**1.05**
% ACCURACY	**103.26**	**98.04**	**103.04**	**98.57**	**103.04**	**98.54**
% STABILITY			**99.79**	**100.54**	**99.79**	**100.50**

6.15.6. Short Term Stock Solution Stability of Analyte at room temperature

The stock solution (Stock ID. ANA02) of Alosetron was divided in two parts. One portion was placed on the bench at room temperature for 20 hours 57 minutes and other portion in refrigerator until analysis. MQC level concentration were prepared from stock solution placed on the bench at room temperature were compared against the freshly prepared stock dilutions at MQC level from other portion stored in the refrigerator. Alosetron was found to be stable in diluent (acetonitrile : water, 10:90) for 20 hours 57 minutes at room temperature with a % stability of 99.57. Results are presented in Table 21.

Table 21: Short-Term Stock Solution Stability of Analyte

Stability Duration : 20 hrs 57 min at room temperature

	Comparison sample peak area	**Stability sample peak area**
STOCK SOLUTION ID.: ANA 02	80265	80447
	80664	80515
	81080	80741
	80192	79438
	80157	79613
	80385	79931
AVERAGE	**80457.167**	**80114.167**
% STABILITY	**99.57**	

6.15.7. Short Term Stock Solution Stability of Internal Standard at room temperature

The stock solution (Stock ID. ISA01) of Alosetron D_3 was divided in two parts. One portion was placed on the bench at room temperature for 20 hours 57 minutes and other portion in refrigerator until analysis. Internal standard working solution were prepared from stock solution placed on the bench at room temperature were compared against the freshly prepared stock dilutions at Internal standard working solution level from other portion stored in the refrigerator. Alosetron D_3 was found to be stable in acetonitrile for 20 hours 57 minutes at room temperature with a % stability of 99.35. Results are presented in Table 22.

Table 22: Short-Term Stock Solution Stability of Internal Standard

Stability Duration : 20 hrs 57 min at room temperature

	Comparison sample peak area	**Stability sample peak area**
STOCK SOLUTION ID.: ISA 01	85337	85499
	86998	86891
	86379	84809
	85449	84937
	86478	85706
	85438	84883
AVERAGE	**86013.167**	**85454.167**
% STABILITY	**99.35**	

6.15.8. Working solution stability in Refrigerator

Alosetron spiking solution (equivalent to LQC and HQC spiking concentrations) and IS spiking solution were prepared using diluent (Acetonitrile: Water) and stored in refrigerator for 64 hours 56 minutes (Stability Samples).

Stability samples of Alosetron were diluted at approximately concentrations equal to LQC and HQC with IS (stability IS) and compared with comparison sample (sample prepared using freshly retrieved stock solution of analyte and IS) are analyzed in a single run, responses were used to determine % stability over time.

Alosetron working solution was found to be stable at refrigerator temperature for 64 hours 56 minutes with a % stability of 98.74 and 99.28 for LQC and HQC respectively. Results are presented in Table 23.

Alosetron D_3 working solution was found to be stable at refrigerator for 64 hours 56 minutes with a % stability of 99.26. Results are presented in Table 24.

Table 23: Working Solution Stability of Analyte in Refrigerator

Stability Duration: 64 hours 56 minutes

	Comparison sample peak area (LQC)	**Stability sample peak area (LQC)**	**Comparison sample peak area (HQC)**	**Stability sample peak area (HQC)**
ANALYTE AREA	3301	3249	156998	156328
	3184	3251	156950	155945
	3292	3235	156320	155522
	3235	3263	155424	154183
	3326	3257	155814	154592
	3329	3164	156536	154709
AVERAGE	**3277.833**	**3236.500**	**156340.333**	**155213.167**
% STABILITY	**98.74**		**99.28**	

Table 24: Working Solution Stability of IS in Refrigerator

Stability Duration : 64 hours 56 minutes

	Comparison sample peak area	**Stability sample peak area**
IS AREA	79788	80245
	80384	80409
	81621	80624
	81009	80031
	80517	79954
	80072	80535
	79786	79728
	80182	79174
	80041	78993
	80370	78927
	79733	78472
	79536	78855
AVERAGE	**80253.250**	**79662.250**
% STABILITY	**99.26**	

6.15.9. Working solution stability in Room temperature

Alosetron spiking solution (equivalent to LQC and HQC spiking concentrations) and IS spiking solution were prepared using diluent (Acetonitrile: Water) and kept at room temperature for 17 hrs 20 minutes (Stability Samples).

Stability samples of Alosetron were diluted at approximately concentrations equal to LQC and HQC with IS (stability IS) and compared with comparison sample (sample prepared using freshly retrieved stock solution of analyte and IS) are analyzed in a single run, responses were used to determine % stability over time.

Alosetron working solution was found to be stable at room temperature for 17 hours 20 minutes with a % stability of 95.85 and 100.26 for LQC and HQC respectively. Results are presented in Table 25.

Alosetron D_3 working solution was found to be stable at room temperature for 17 hours 20 minutes with a % stability of 100.23. Results are presented in Table 26.

Table 25: Working Solution Stability of Analyte in Room Temperature

Stability Duration : 17 hours 20 minutes

	Comparison sample peak area (LQC)	**Stability sample peak area (LQC)**	**Comparison sample peak area (HQC)**	**Stability sample peak area (HQC)**
ANALYTE AREA	3486	3340	152382	153467
	3527	3214	153205	153627
	3422	3278	153510	152985
	3342	3308	152675	153508
	3303	3228	153787	153344
	3447	3308	151300	152274
AVERAGE	**3421.167**	**3279.333**	**152809.833**	**153200.833**
% STABILITY	**95.85**		**100.26**	

Table 26: Working Solution Stability of IS in Room Temperature

Stability Duration : 17 hours 20 minutes

	Comparison sample peak area	**Stability sample peak area**
IS AREA	75735	76455
	76986	76900
	76450	75980
	76128	76739
	76014	76887
	76908	77402
	76722	76439
	76251	76331
	76349	75051
	75795	76207
	75757	76310
	75292	75808
AVERAGE	**76198.917**	**76375.750**
% STABILITY	**100.23**	

6.15.10. Long Term Stock Solution Stability of Analyte in refrigerator

Alosetron stock solution stability in refrigerator was performed at 18th day (Stability Samples). Freshly prepared stock solution of Alosetron (Comparison Samples) and stability samples were diluted to approximately MQC concentration and analyzed in a single run, Analyte responses were used to determine % stability over time. Alosetron was found to be stable in diluents (ACN: Water 10:90 % v/v) for 18 days. The % stability of Alosetron at 18th day was found to be 100.67. Results are presented in Table 27.

Table 27: Long Term Stock Solution Stability of Analyte at 18 days

Storage Condition: Refrigerator (2- 8 ˚C)

Analyte	**Concentration of stability sample**	**Concentration of fresh sample**
ANA02	90.343 µg/mL	91.058 µg/mL

	Fresh Solution Area	**Stability Solution Area**
STOCK SOLUTION ID.: ANA02	83485	83793
	84336	83703
	83312	82492
	83953	83558
	84134	84275
	82554	83364
AVERAGE	**83629.000**	**83530.833**
% STABILITY	**100.67**	

6.15.11. Long Term Stock Solution Stability of Internal Standard in refrigerator

Alosetron D_3 stock solution stability in refrigerator was performed at 18th day (Stability Samples). Freshly prepared stock solution of Alosetron D_3 (Comparison Samples) and stability samples were diluted to approximately same concentration and analyzed in a single run, responses were used to determine % stability over time. Alosetron D_3 was found to be stable in Acetonitrile for 18 days. The % stability of Alosetron D_3 at 18th day was found to be 97.99. Results are presented in Table 28.

Table 28: Long Term Stock Solution Stability of Internal Standard at 18 days

Storage Condition: Refrigerator (2- 8 ˚C)

Analyte	**Concentration of stability sample**	**Concentration of fresh sample**
ISA-01	101.559 µg/mL	99.889 µg/mL

	Fresh Solution Area	**Stability Solution Area**
STOCK SOLUTION ID.: ISA01	110820	110921
	112016	111539
	111062	109241
	111120	110891
	111252	110717
	109815	110316
AVERAGE	**111014.167**	**110604.167**
% STABILITY	**97.99**	

6.15.12. Long Term Stability of Analyte in Matrix at -20°C and -70°C

Long term stability of analyte in K_2EDTA plasma was performed by using six replicates of LQC and HQC samples stored at -20°C and -70°C on 17th days of storage (Stability Samples) The samples were analysed with freshly prepared calibration standards and quality control samples (Comparison Samples). Alosetron back calculated concentrations were used to determine % stability over time. Alosetron was found to be stable in plasma for 17 days at -20°C and -70°C.

The % Stability of Alosetron in K_2EDTA plasma after 17 days of storage at -20°C and -70°C was found to be 112.14, 108.90 for LQC, 96.38 and 96.59 for HQC samples respectively. Results are presented in Table 29.

Table 29 : Long -Term Stability of Analyte in Matrix at 17 days

Standard	**CS 01**	**CS 02**	**CS 03**	**CS 04**	**CS 05**	**CS 06**	**CS 07**	**CS 08**	**Slope**	**Intercept**	**r^2**
Nominal Conc.(ng/ml)	**0.160**	**0.320**	**0.620**	**5.820**	**11.640**	**17.220**	**22.960**	**28.700**			
Back Calc.Conc. (ng/ml):	0.159	0.327	0.611	5.939	11.575	17.279	22.934	28.212	0.0812	-0.0005	0.9997
% ACCURACY	**99.28**	**102.08**	**98.63**	**102.05**	**99.44**	**100.34**	**99.89**	**98.30**			

QC ID	**Comparison Sample**		**Stability Sample**			
			-70 ˚C		**-20 ˚C**	
	LQC	**HQC**	**LQC**	**HQC**	**LQC**	**HQC**
Nominal Concentration (ng/ml)	**0.460**	**22.580**	**0.460**	**22.760**	**0.460**	**22.760**
Back Calculated Concentration (ng/ml)	0.427	22.988	0.478	22.087	0.462	22.377
	0.440	22.937	0.479	22.412	0.481	22.456

	0.434	22.912	0.473	22.557	0.467	22.269
	0.439	22.982	0.458	22.247	0.463	22.283
	0.454	23.280	0.479	22.827	0.605	22.832
	0.436	23.149	0.493	22.467	0.469	22.090
AVERAGE	**0.438**	**23.041**	**0.477**	**22.433**	**0.491**	**22.385**
SD	**0.009**	**0.143**	**0.011**	**0.256**	**0.056**	**0.250**
% CV	**2.05**	**0.62**	**2.31**	**1.14**	**11.41**	**1.12**
% ACCURACY	**95.22**	**102.04**	**103.70**	**98.56**	**106.74**	**98.35**
% STABILITY			**108.90**	**96.59**	**112.10**	**96.38**

6.15.13. Stability of Analyte in Blood

Stability of Alosetron in blood was evaluated at room temperature. Two sets (each six replicates) of low and high quality control samples were spiked in blood samples and one set of samples immediately centrifuged and plasma was harvested (Comparison samples).

Another set of QC samples kept at room temperature for approximately 02 hours 06 minutes and plasma was harvested after centrifugation, all the samples were processed as per method SOP and analyzed in a single batch. The % stability in blood was calculated using response, found to be stable in human blood for 02 hours 06 minutes at room temperature with % stability of 100.00 and 99.71 for LQC and HQC respectively. Results are presented in Table 30.

Table 30: Stability of Analyte in Blood

Stability Duration : 2 hour 06 minutes

QC ID	Comparison Sample Area Ratio		Stability Sample Area Ratio	
	LQC	HQC	LQC	HQC
ANALYTE RESPONSE	0.041	2.071	0.045	2.053
	0.047	2.075	0.041	2.081
	0.041	2.114	0.044	2.145
	0.044	2.099	0.042	2.072
	0.040	2.065	0.041	2.039
	0.043	2.141	0.043	2.138
AVERAGE	**0.043**	**2.094**	**0.043**	**2.088**
SD	**0.003**	**0.030**	**0.002**	**0.044**
%CV	**6.98**	**1.43**	**4.65**	**2.11**
% STABILITY			**100.00**	**99.71**

6.16 SOP DEVIATIONS

There were no significant deviations observed.

6.17 CONCLUSION

A selective and sensitive Liquid Chromatography-Mass Spectrometry/Mass Spectrometry method to quantitate Alosetron in K_2EDTA human plasma over the concentration range 0.160 to 28.920 ng/mL was successfully validated. This method is suitable for sample analysis to support bioequivalence/bioavailability and/or pharmacokinetic studies of Alosetron.

Chronology of validation runs are shown in Table 31.

Table 31: Chronology of Validation Runs

Acquisition Date	Validation Parameters	Batch ID	Instrument ID	Status	Reason for Failure
13/08/13	Selectivity	SPE&SEL	BA-MS-07	Accepted	NA
13/08/13	Matrix Effect	ME	BA-MS-07	Accepted	NA
14/08/13	Precision and Accuracy Batch 1	PA 01	BA-MS-07	Accepted	NA
14/08/13	Carry Over Test	ASCOT	BA-MS-07	Accepted	NA
14/08/13	Precision and Accuracy Batch 2 & Recovery	PA02 & REC	BA-MS-07	Accepted	NA
14/08/13	Carry Over Test	ASCOT	BA-MS-07	Accepted	NA
14/08/13	Ruggedness	RUG	BA-MS-08	Accepted	NA
14/08/13	Carry Over Test	ASCOT	BA-MS-08	Accepted	NA
14/08/13	Short Term Stock Solution Stability	STSS	BA-MS-08	Accepted	NA
15/08/13	Reinjection Reproducibility	RIR	BA-MS-08	Accepted	NA
15/08/13	Working Solution Stability in Room Temperature	WSS_RT	BA-MS-08	Accepted	NA
15/08/13	Precision and Accuracy Batch 3 & Dilution Integrity	PA 03	BA-MS-08	Not Accepted	% CV for LLOQ samples not within the limit
15/08/13	Production Batch Precision & Accuracy	PBPA	BA-MS-07	Not Accepted	% CV for LQC samples not within the limit
16/08/13	Precision and Accuracy Batch 4 & Dilution Integrity	PA 04	BA-MS-08	Accepted	NA
16/08/13	Freeze Thaw Stability at -70ºC and -20 ºC (FTS)	STABILITY 1	BA-MS-08	Accepted	NA
	Bench Top Stability (BTS)	STABILITY 1	BA-MS-08	Accepted	NA
16/08/13	Wet Extract Stability at Room	STABILITY 2	BA-MS-07	Accepted	NA

	Temperature (WES_RT)				
	Wet Extract Stability at Refrigerator (WES_RF)	STABILITY 2	BA-MS-07	Accepted	NA
	Auto Sampler Stability (ASS)	STABILITY 2	BA-MS-07	Not Accepted	% CV & Stability for LQC samples not within the limit
16/08/13	Production Batch Precision & Accuracy	PBPA	BA-MS-07	Accepted	NA
17/08/13	Working Solution Stability in Refrigerator	WSS_RF	BA-MS-08	Accepted	NA
17/08/13	Auto Sampler Stability (ASS)	STABILITY 3	BA-MS-08	Accepted	NA
17/08/13	Analyte Stability in Blood	ASB	BA-MS-08	Accepted	NA
30/08/13	Long term stock solution stability- 18 days	LTSS	BA-MS-08	Accepted	NA
30/08/13	Long Term Stability of Analyte in Matrix- 17 days	LTMS	BA-MS-08	Accepted	NA

ACCEPTANCE CRITERIA

Experiment	Acceptance Criteria
Carry Over Test	o Peak response obtained in the blank sample at the retention time of analyte and IS should be less than 20 % and 5% of mean LLOQ response.
Selectivity	o Response of the interfering peaks at the retention time of analyte(s) should be ≤20% of the response of analyte(s) in LLOQ standard. o Response of the interfering peaks at the retention time of IS should be ≤5% of IS responses in LLOQ Standard. o At least 80% of the screened matrix lots should meet the acceptance criteria including lipimic and haemolysed lot.
Matrix effect	o CV % of the analyte matrix factor and IS normalized matrix factor calculated from different lots of matrix should not be greater than 15% at each level.
Sensitivity	o Limit of Quantification is acceptable if mean of 6 determinations is within ± 20% of the nominal concentration and precision (CV) is ≤ 20%. o Signal to noise shall be more than 5.
Linearity	o Percentage interference at the retention time of analyte(s) in any of duplicates of blank matrix and zero standards should not be more than 20% of the accepted LLOQ response.

	o Percentage interference at the retention time of the internal standard in any of duplicates of blank matrix should not be more than 5% of the mean of the accepted CC standards IS response. o Accuracy (% Nominal) of calibration standards should be within ± 15% from the nominal concentration other than LLOQ where it should be within ± 20%. o At least 75% or a minimum of 6 non-zero standards should meet the given acceptance criteria including LLOQ and ULOQ. o Correlation coefficient (r) should be ≥0.99 or coefficient of determination (r2) should be ≥0.98.
Precision & Accuracy	o Back calculated values of at least 67% (20 out of 30) of total QC samples (4 out of 6) at each level (LQC,IMQC, MQC, & HQC) should be within ±15% of the nominal concentration except LLOQ where it should be within ± 20%. o Accuracy: within and between batch mean concentration (% Nominal) should be within ± 15% at each level except LLOQ where it should be within ± 20%. o Precision: within and between day batch precision (% CV) for all QC concentrations should be ≤ 15% except LLOQ where it should be ≤ 20%.
Recovery	o Recovery of analyte(s) is acceptable if % CV is ≤ 15% for low, middle and high QC concentration, individually and totally. o Recovery of internal standard is acceptable if % CV is ≤ 15% o Recovery should not be more than 115%.
Dilution Integrity	o The DI is acceptable if the mean % nominal is within ± 15% and CV is ≤ 15%. o 67% of total DI samples should be within ± 15% of nominal value.
Ruggedness	o As given for Precision & Accuracy
Re-injection Reproducibility	o Back calculated values of at least 67% of total QC samples (4 out of 6) at each level should be within ±15% of the nominal concentration. o Precision: CV for QC concentrations should be ≤ 15%.
Production Batch P&A	o Back calculated values of at least 67% of total QC samples at each level should be within ±15% of the nominal concentration. o Precision: CV for QC concentrations should be ≤ 15%.
Stability	
Stock solution stability	o Percentage stability of analyte(s) and IS stocks should be within the range of 90 - 110% for both short term and long term stock solution stability
Working Solution Stability	o Percentage stability of analyte(s) and IS working solutions should be within the range of 90 - 110%.
Auto Sampler stability	o Auto sampler stability should be within 85-115% and precision (CV) should be ≤15% at HQC and LQC levels. o At least 67% of QCs per level must be within 85-115%.
Wet Extract stability	o The wet extract Stability of the Analyte(s) should be within 85-115% and precision (CV) should be ≤ 15% at HQC and LQC levels. o At least 67% of QCs per level must be within 85-115%.
Dry Extract Stability	o The dry Extract Stability of the Analyte(s) should be within 85-115% and precision (CV) should be ≤ 15% at HQC and LQC levels. o At least 67% of QCs per level must be within 85-115%.

Bench Top Stability	o Bench top stability of the analyte(s) should be within 85-115% and precision (CV) should be ≤ 15% at LQC and HQC levels. o At least 67% of QCs per level must be within 85-115%.
Freeze Thaw Stability	o Freeze-Thaw Stability of the Analyte(s) should be within 85-115% and precision (CV) should be ≤ 15% at HQC and LQC levels. o 67% of total Comparison samples must be 85-115%.
Stability of Analyte in Blood	o The stability of the analyte(s) should be within the range of 85-115% o 67% of total Comparison samples must be 100 ± 15% nominal value. o CV of should be ≤ 15% at HQC and LQC levels.
Long Term stability in Matrix	o Long term stability in matrix of the analyte(s) should be within the range of 85-115%. o 67% of total Comparison samples must be 100 ± 15% nominal value. o CV of should be ≤ 15% at HQC and LQC levels.

CHROMATOGRAMS

CHROMATOGRAMS

Figure 1. Representative chromatogram of processed blank matrix.

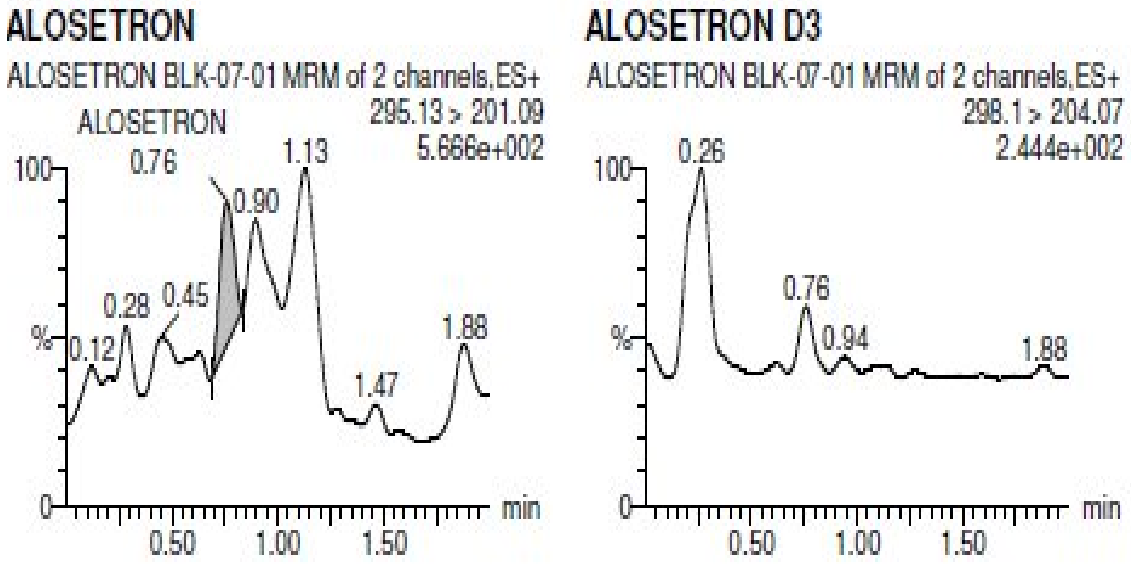


Figure 2. Representative chromatogram of lower limit of quantification sample containing Alosetron (0.160 ng/mL) in matrix

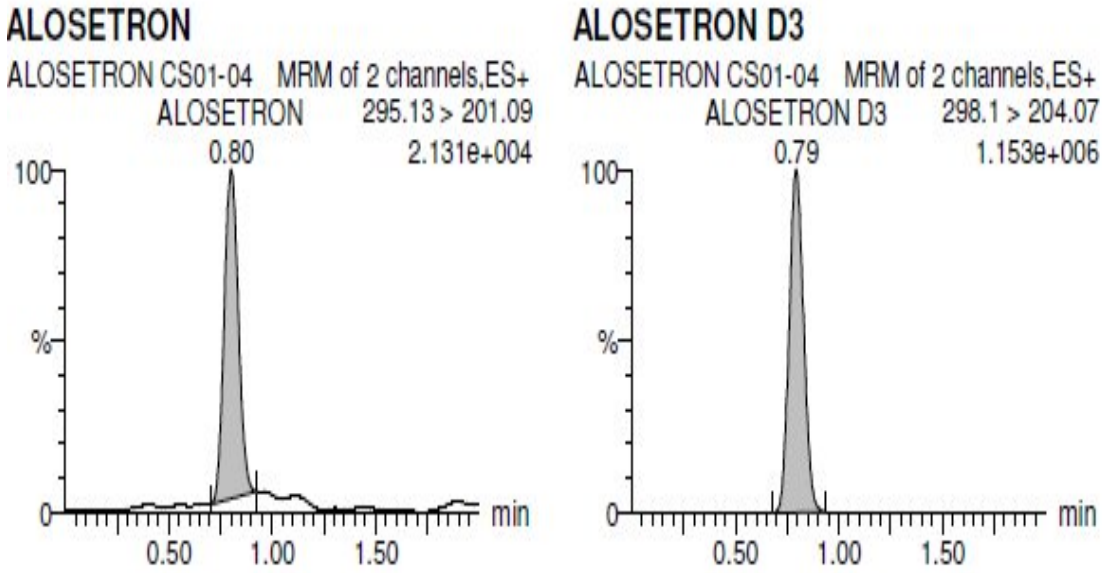


Figure 3. Representative chromatogram of low quality control sample containing Alosetron (0.460 ng/mL) in matrix.

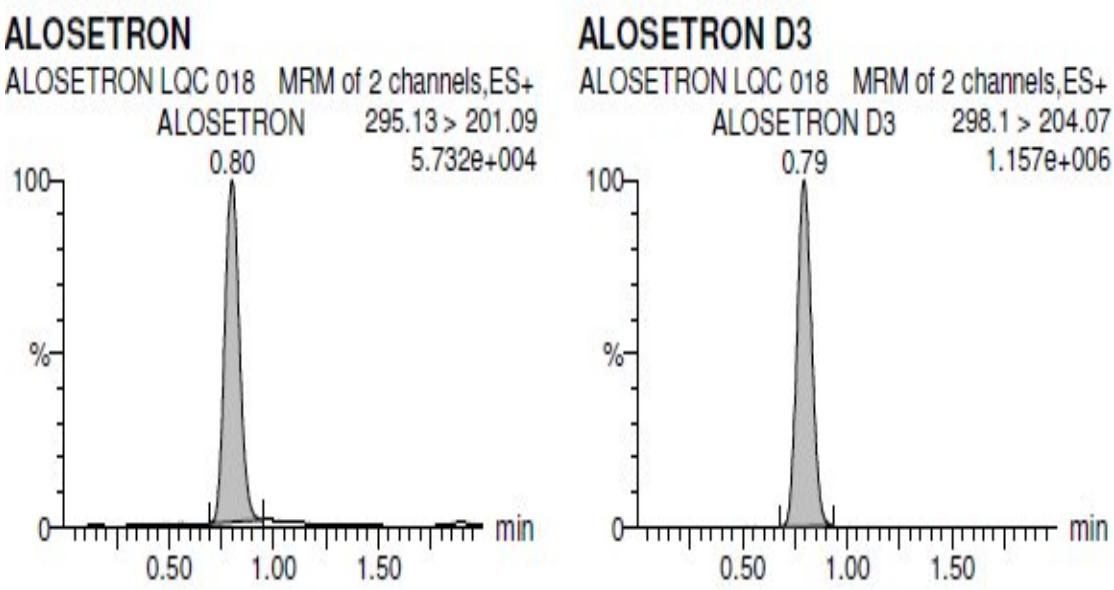


Figure 4. Representative chromatogram of medium quality control sample containing Alosetron (9.100 ng/mL) in matrix.

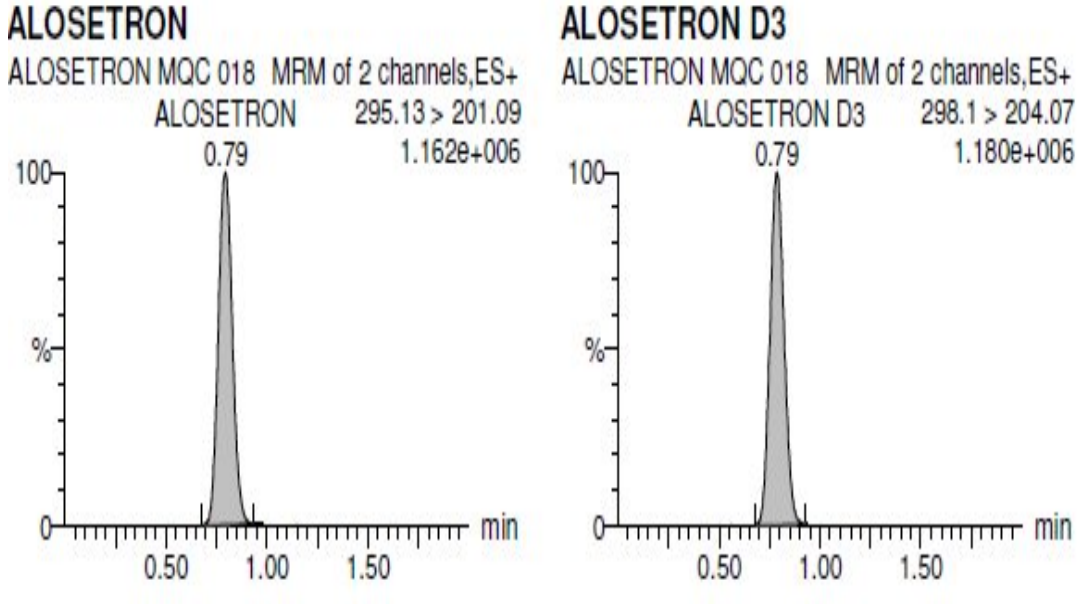


Figure 5. Representative chromatogram of high quality control sample containing Alosetron (22.760 ng/mL) in matrix.

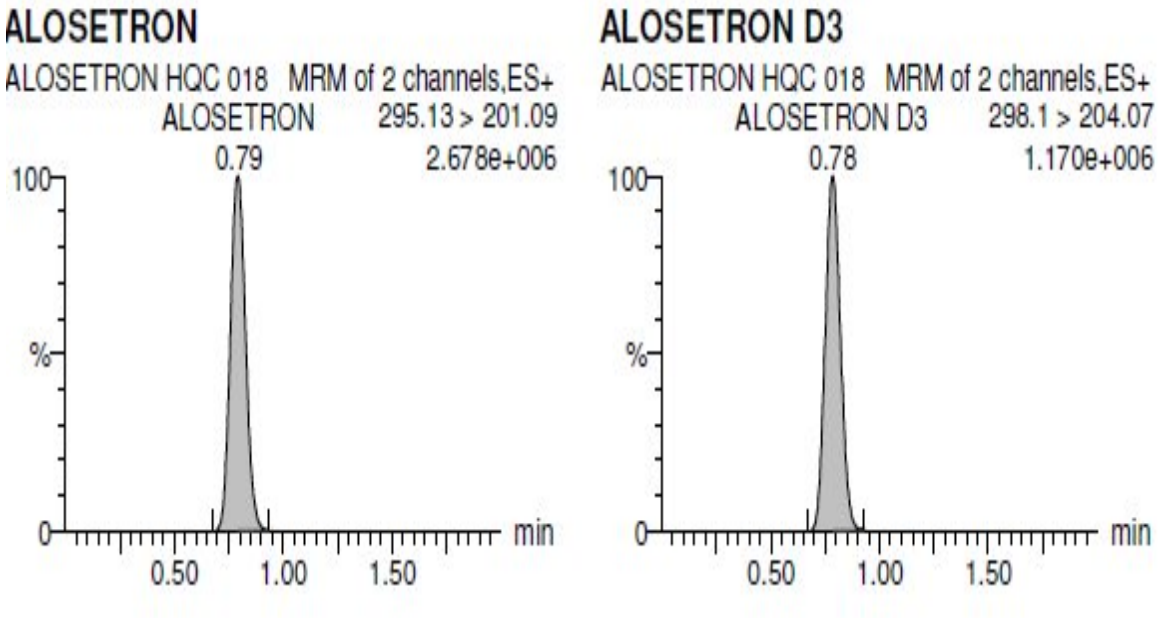


Figure 6. Representative chromatogram of upper limit of quantification sample containing Alosetron (28.920 ng/mL) in matrix.

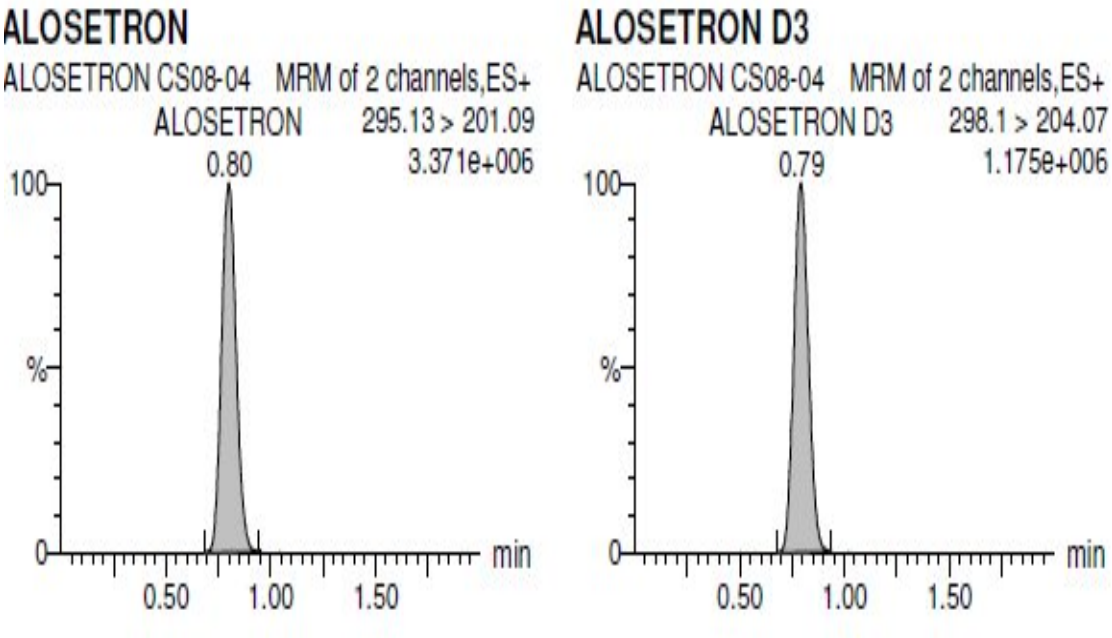
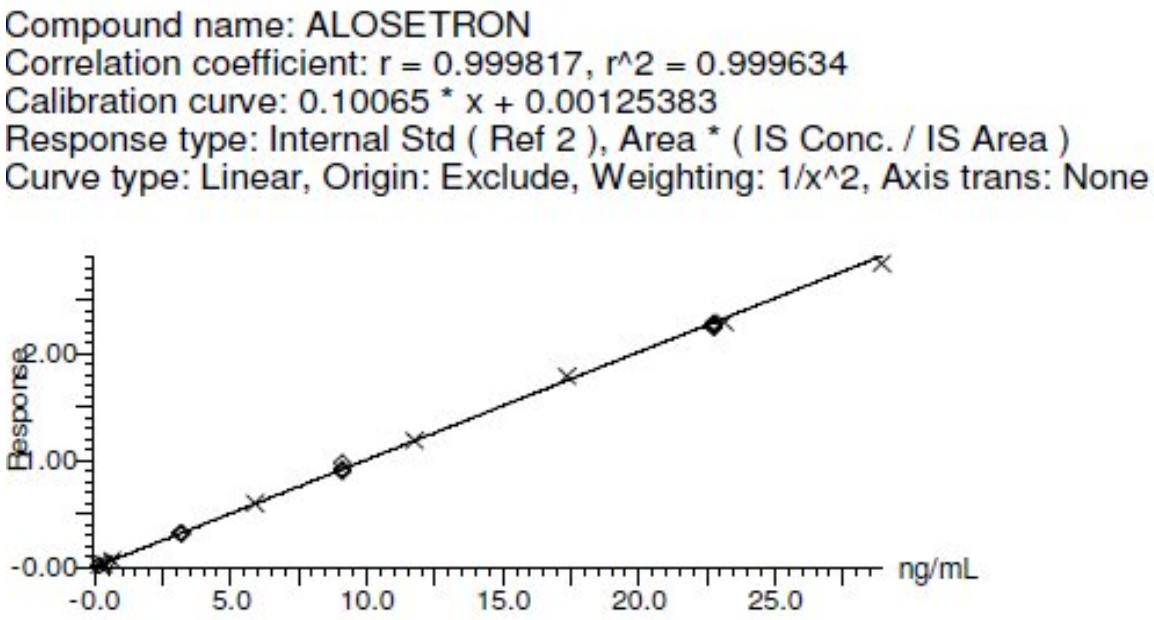


Figure 7. Representative calibration curve for Alosetron in matrix



SUMMARY AND CONCLUSION

SUMMARY

A LC-MS/MS method for the determination of Alosetron in human K_2EDTA plasma is described in method SOP No. BM-020-02. The method was validated according to SOP NO.: EBL-BA-24-02.

Parameters	Results
Calibration curve Range	0.160 to 28.920 ng/mL
Coefficient of determination (r^2)	Greater than 0.9991
Between-Run Accuracy	QC % nominal concentrations: 98.58 to 108.13 %
Between-Run Precision	QC coefficient of variation: 0.66 to 10.98 %
Within- Run Accuracy	QC % nominal concentrations: 98.13 to 118.13 %
Within- Run Precision	QC coefficient of variation: 0.35 to 12.70 %
Selectivity	92.3 % & 100 % of tested matrix was within the limit for Analyte and IS
Sensitivity (LLOQ)	0.160 ng/mL
Sensitivity -Accuracy	QC % nominal concentrations: 99.38 %
Sensitivity-Precision	QC coefficient of variation: 1.26 %
Dilution Integrity- Accuracy (1/2 and 1/4)	QC % nominal concentrations: 99.53 and 98.81 %
Dilution Integrity-Precision (1/2 and 1/4)	QC coefficients of variation; 1.12 and 0.48 %
Matrix Effect (LQC & HQC)	Analyte – 2.43 and 1.59 %. IS normalized – 4.49 and 3.18 %
Ruggedness- Accuracy	QC % nominal concentrations: 99.70 to 107.50 %
Ruggedness- Precision	QC coefficients of variation; 0.53 to 2.91 %
Recovery - Analyte	LQC-77.36 %, MQC-74.39 %, HQC-75.37 % and Global-
Recovery - IS	79.34 %
Auto sampler Stability (LQC & HQC)	Stability after 21 h 40 min : 99.79 & 100.14 %
Wet Extract Stability in Room temperature (LQC &	Stability after 25 h 45 min : 91.06 & 96.48 %
Wet Extract Stability in Refrigerator (LQC & HQC)	Stability after 46 h 44 min : 95.93 & 95.88 %
Freeze Thaw Stability (-20 ± 5°C) (LQC & HQC)	Stability after 4 cycles: 99.79 & 100.50 %
Freeze Thaw Stability (-70 ± 20 °C) (LQC & HQC)	Stability after 4 cycles: 99.79 & 100.54 %
Short-Term Stock Solution Stability - Analyte	Stability after 20 h 57 min : 99.57 %
Short-Term Stock Solution Stability - IS	Stability after 20 h 57 min : 99.35 %
Working solution stability in Room temperature -	Stability after 17 h 20 min : LQC 95.85 & HQC 100.26
Working solution stability in Room temperature - IS	Stability after 17 h 20 min : 100.23 %
Working solution stability in Refrigerator - Analyte	Stability after 64 h 56 min: LQC 98.74 & HQC 99.28 %
Working solution stability in Refrigerator - IS	Stability after 64 h 56 min: 99.26 %
Bench top stability(LQC & HQC)	Stability after 16 h 25 min : LQC 101.95 & HQC 101.05
Long term stock solution stability- Analyte	Stability after 18 days : 100.67%
Long term stock solution stability- IS	Stability after 18 days : 97.99 %
Long Term stability of analyte in K_2EDTA Plasma (-	Stability after 17 days: LQC 112.10 & HQC 96.38 %
Long Term stability of analyte in K_2EDTA Plasma (-	Stability after 17 days: LQC 108.90 & HQC 96.59 %
Stability of Analyte in Blood	Stability after 2 h 06 min LQC 100.00 & HQC 99.71 %

Alosetron is extracted from an aliquot of human K_2EDTA plasma using Protein precipitation method and injected into a liquid chromatography equipped with tandem mass spectrometry detector. Quantitation was done by peak area ratio method. A weighted (1/X2) linear regression is

performed to determine the concentration of the analyte. Results obtained from this validation are presented in Tables 1-31. This method demonstrates acceptable performance as outlined by EBL SOPs and is suitable for the determination of Alosetron in human K_2EDTA plasma over the range 0.160 to 28.920 ng/mL.

CONCLUSION

The proposed method demonstrates good stability conditions for drug in biological matrix.

This method is beneficial in the following aspects

- Less expensive
- Less matrix interference

The proposed sample preparation technique gives precise, accurate and reproducible response with a considerably short term analysis.

A selective and sensitive Liquid Chromatography-Mass Spectrometry/Mass Spectrometry method to quantitate Alosetron in K_2EDTA human plasma over the concentration range from 0.160 to 28.920 ng/ml was successfully validated. This method is suitable for sample analysis to support bioequivalence/bioavailability and/or pharmacokinetic studies of Alosetron.

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